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(71) Applicant *(for all designated States except US)*: CAMBIA BIOSYSTEMS LLC [US/US]; 1150 – 114th Avenue S.E., Bellevue, WA 98009-9722 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: JEFFERSON, Richard, A. [US/AU]; 135 Wickerslack Lane, Googong, NSW 2620 (AU). KILIAN, Andrzej [PL/AU]; 42 Campbell Street, Ainslie, ACT 2602 (AU). KEESE, Paul, Konrad [AU/AU]; 10 Morehead Street, Curtin, ACT 2605 (AU).

(74) Agents: MCMASTERS, David, D. et al.; Seed and Berry LLP, 6300 Columbia Center, 701 Fifth Avenue, Seattle, WA 98104-7092 (US).

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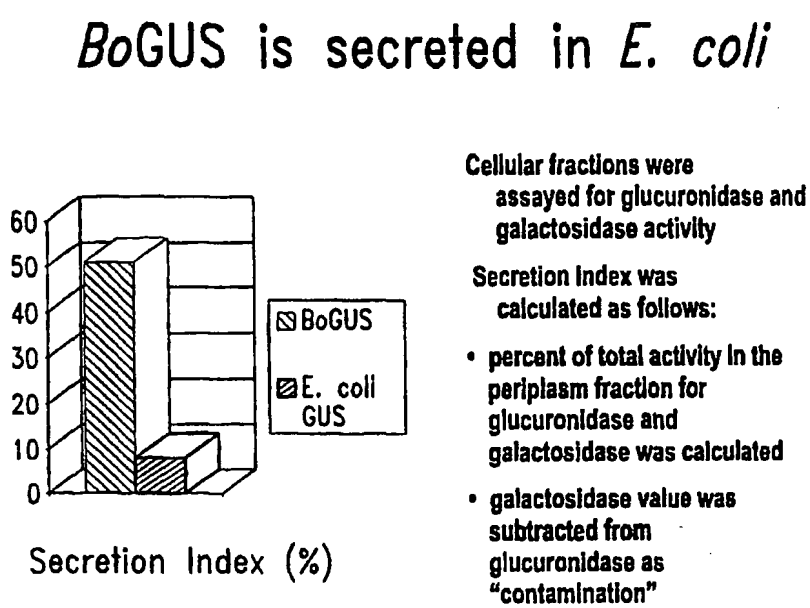

(57) Abstract

Genes encoding microbial β-glucuronidase and protein that is secreted and its uses are provided.

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DESCRIPTION

MICROBIAL β-GLUCURONIDASE GENES, GENE PRODUCTS AND USES THEREOF

TECHNICAL FIELD

The present invention relates generally to forms of microbial β-glucuronidase that are directed to specific cell compartments, and more specifically to a secreted form of β-glucuronidase and uses of these β-glucuronidases thereof.

BACKGROUND OF THE INVENTION

The natural habitat of *E. coli* is the gut, and the β-glucuronidase (GUS) activity of *E. coli* plays a specific and very important role in its natural history. The gut is a rich source of glucuronic acid compounds, providing a carbon source that can be efficiently exploited by *E. coli*. Glucuronide substrates are taken up by *E. coli* via a specific transporter, the glucuronide permease (U.S. Patent No. 5,288,463 and 5,432,081), and cleaved by β-glucuronidase. The glucuronic acid residue thus released is used as a carbon source. In general, the aglycon component of the glucuronide substrate is not used by *E. coli* and passes back across the bacterial membrane into the gut to be reabsorbed into the bloodstream and undergo glucuronidation in the liver, which begins the cycle again.

In *E. coli*, β-glucuronidase is encoded by the gusA gene (Novel and Novel, *Mol. Gen. Genet. 120*:319-335, 1973), which is one member of an operon comprising three protein-encoding genes. The second gene, gusB, encodes a specific permease (PER) for β-glucuronides. The third gene, gusC, encodes an outer membrane protein (MOP) of approximately 50 kDa that facilitates access of glucuronides to the permease located in the inner membrane. The principle repressor for the GUS operon, gusR, maps immediately upstream of the operon.

β-glucuronidase activity is expressed in almost all tissues of all vertebrates and many mollusks (Levvy and Conchie, 1966). In addition, the free-living soil nematode, *Caenorhabditis elegans,* has an endogenous ß-glucuronidase activity (Sebastiani et al, 1987; Jefferson et al, 1987), which occurs at low levels in the intestine of the worm. The enzyme has been purified from many mammalian sources (*e.g.* Tomino et al, 1975) and forms a homotetrameric structure with a subunit molecular weight of approximately 70 kDa.

The vertebrate enzyme is synthesized with a signal sequence at the amino terminus, then transported to and glycosylated within the endoplasmic reticulum, and ultimately localized intracellularly within vacuoles. If any of the mammalian enzyme is secreted, it probably contributes little to the total activity as the enzyme is relatively unstable. Thus, for use in medical diagnostics (*e.g.*, drug testing) and transgenic constructions, the *E. coli* enzyme is preferred because it is much more active and stable than the mammalian enzyme against most biosynthetically derived ß-glucuronides (Tomasic and Keglevic, 1973; Levvy and Conchie, 1966).

Production of GUS for use in *in vitro* assays, such as medical diagnostics, is costly and requires extensive manipulation as GUS must be recovered from cell lysates. A secreted form of GUS would reduce manufacturing expenses, however, attempts to cause secretion have been unsuccessful. In addition, for use in transgenics, the current GUS system has somewhat limited utility because enzymatic activity is detected intracellularly by deposition of toxic colorimetric products during the staining or detection of GUS. Moreover, in cells that do not express a glucuronide permease, the cells must be permeabilized or sectioned for introduction of the substrate. Thus, this conventional staining procedure generally results in the destruction of the stained cells. In light of this limitation, a secreted GUS would allow for development of non-destructive marker system, especially useful for agricultural field work.

The present invention provides gene and protein sequences of secreted β-glucuronide, variants thereof, and use of the protein as a transformation marker, while providing other related advantages.

SUMMARY OF THE INVENTION

In one aspect, an isolated nucleic acid molecule is provided comprising a nucleic acid sequence encoding a secreted form of β-glucuronidase, wherein the nucleic acid sequence comprises the amino acid sequence as presented in Figure 3 or hybridizes under stringent conditions to the complement of the sequence comprising nucleotides 1662-3467 of Figure 1 and which encodes a functional β-glucuronidase. In preferred embodiments, the nucleic acid molecule comprises nucleotides 1662-3467 of Figure 1 or encodes the amino acid sequence of Figure 3, or a variant thereof.

In another aspect, the invention provides an isolated secreted form of β-glucuronidase, wherein β-glucuronidase is encoded by the isolated nucleic acid molecule or by a nucleic acid molecule that hybridizes under stringent conditions to the complement of nucleotides 1662-3467 of Figure 1 and which encodes a functional β-glucuronidase. In a preferred embodiment, the isolated secreted form of β-glucuronidase comprises the amino acid sequence of Figure 3, or a variant thereof.

The invention also provides vectors and host cells, comprising a nucleic acid molecule encoding a secreted form of β-glucuronidase, wherein the β-glucuronidase sequence is in operative linkage with a promoter element. In preferred embodiments, the promoter element is a promoter derived from a plant pathogen. Preferred host cells are selected from the group consisting of a plant cell, an insect cell, a fungal cell, an animal cell and a bacterial cell.

The invention also provides a method of producing a secreted form of β-glucuronidase, comprising: (a) introducing a vector according to any one of claims 6-13 into a host cell, wherein the vector comprises nucleic acid sequence encoding the β-glucuronidase in an expressible form; and (b) growing the host cell under conditions wherein the β-glucuronidase is expressed. The method may further comprise isolating the β-glucuronidase from cell supernatant or periplasm.

In other aspects, the invention provides methods of introducing a controller element into a host cell, monitoring expression of a gene of interest or a portion thereof in a host cell, monitoring activity of a controller element in a host cell,

transforming a host cell with a gene of interest or portion thereof, and positive selection for a transformed cell.

In other aspects, transgenic cells are provided, such as plant cells, insect cells, and transgenic plants and insects.

In other aspects, kits comprising microbial GUS are provided.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe in more detail certain procedures or compositions (*e.g.*, plasmids, etc.), and are therefore incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A & 1B is DNA sequence of an approximately 6 kb fragment that encodes β-glucuronidase from *Bacillus*.

Figure 2 is a schematic of the DNA sequence of a *Bacillus* 6 kb fragment showing the location and orientation of the major open reading frames. S-GUS is β-glucuronidase.

Figure 3 is an amino acid sequence of *Bacillus* GUS.

Figure 4A-4C is a DNA sequence of *Bacillus* GUS with the predicted amino acid translation.

Figure 5 presents amino acid alignments of *Bacillus* GUS (BGUS) *E. coli* GUS (EGUS) and human GUS (HGUS).

Figure 6 is a graph showing that *Bacillus* GUS is secreted in *E. coli* transformed with an expression vector encoding *Bacillus* GUS. The secretion index is the percent of total activity in periplasm less the percent of total β-galactosidase activity in periplasm.

Figure 7 is a graph illustrating the half-life of *Bacillus* GUS and *E. coli* GUS at 65°C.

Figure 8 is a graph showing the turnover number of *Bacillus* GUS and *E. coli* GUS enzymes at 37°C.

Figure 9 is a graph showing the turnover number of *Bacillus* GUS and *E. coli* GUS enzymes at room temperature.

Figure 10 is a graph presenting relative enzyme activity of *Bacillus* GUS in various detergents.

Figure 11 is a graph presenting relative enzyme activity of *Bacillus* GUS in the presence of glucuronic acid.

Figure 12 is a graph presenting relative enzyme activity of *Bacillus* GUS in various organic solvents and in salt.

Figure 13A-13E is a DNA sequence of *Bacillus* GUS that is codon optimized for production in *E. coli*.

Figure 14 is a schematic of the DNA sequence of *Bacillus* GUS that is codon optimized for production in *E. coli*.

Figure 15 is a map of the expression vector pLAD-F48 containing *Bacillus* GUS, showing key features.

DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms that will be used hereinafter.

As used herein, "β-glucuronidase" refers to an enzyme that catalyzes the hydrolysis of β-glucuronides. For assays to detect β-glucuronidase activity, fluorogenic or chromogenic substrates are preferred. Such substrates include, but are not limited to, p-nitrophenyl β-D-glucuronide and 4-methylumbelliferyl β-D-glucuronide. Assays and some exemplary substrates for determining β-glucuronidase activity, also known as GUS activity, are provided in U.S. Patent No. 5,268,463.

As used herein, a "secreted form of a microbial β-glucuronidase" refers to a microbial β-glucuronidase that is capable of being localized to an extracellular environment of a cell, including extracellular fluids, periplasm, or membrane bound on the external face of a cell but not bound as an integral membrane protein. Some of the protein may be found intracellularly. Thus, secreted microbial GUS encompasses GUS proteins that are secreted in *E. coli* statistically significantly more than EcGUS (*E. coli*

GUS). The amino acid and nucleotide sequences of an exemplary secreted β-glucuronidase are presented in Figure 1 and SEQ ID NOs: 1 and 2. Secreted microbial GUS also encompasses variants of β-glucuronidase. A variant may be a portion of the secreted β-glucuronidase and/or have amino acid substitutions, insertions, and deletions, either found naturally as a polymorphic allele or constructed.

As used herein, "percent sequence identity" is a percentage determined by the number of exact matches of amino acids or nucleotides to a reference sequence divided by the number of residues in the region of overlap. Within the context of this invention, preferred amino acid sequence identity for a variant is at least 75% and preferably greater than 80%, 85%, 90% or 95%. A nucleotide variant will typically be sufficiently similar in sequence to hybridize to the reference sequence under stringent hybridization conditions (for nucleic acid molecules over about 500 bp, conditions include a solution comprising about 1 M Na+ at 25° to 30°C below the Tm; *e.g.*, 5 x SSPE, 0.5% SDS, at 65°C; *see*, Ausubel, et al., *Current Protocols in Molecular Biology*, Greene Publishing, 1995; Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, 1989). Some variants may not hybridize to the reference sequence because of codon degeneracy, such as introduced for codon optimization in a particular host, in which case amino acid identity may be used to assess similarity of the variant to the reference protein.

As used herein, a "glucuronide" or "β-glucuronide" refers to an aglycon conjugated in a hemiacetal linkage, typically through the hydroxyl group, to the C1 of a free D-glucuronic acid in the β configuration. β-glucuronides consist of virtually any compound linked to the 1-position of glucuronic acid as a beta anomer, and are typically, though by no means exclusively, found as the -O-glycoside. β-glucuronides are produced naturally in most vertebrates through the action of UDP-glucuronyl transferase as a part of the process of solubilizing, detoxifying, and mobilizing both natural and xenobiotic compounds, thus directing them to sites of excretion or activity through the circulatory system.

β-glucuronides in polysaccharide form are also common in nature, most abundantly in vertebrates, where they are major constituents of connective and

lubricating tissues in polymeric form with other sugars such as N-acetylglucosamine (*e.g.*, chondroitan sulfate of cartilage, and hyaluronic acid, which is the principle constituent of synovial fluid and mucus). β-glucuronides are relatively uncommon or absent in plants. Glucuronides and galacturonides found in plant cell wall components (such as pectin) are generally in the alpha configuration, and are frequently substituted as the 4-O-methyl ether; hence, such glucuronides are not substrates for β -glucuronidase.

An "isolated nucleic acid molecule" refers to a polynucleotide molecule in the form of a separate fragment or as a component of a larger nucleic acid construct, that has been separated from its source cell (including the chromosome it normally resides in) at least once in a substantially pure form. Nucleic acid molecules may be comprised of a wide variety of nucleotides, including DNA, RNA, nucleotide analogues, or some combination of these.

Microbial glucuronidase genes and gene products

As noted above, this invention provides gene sequences and gene products for secreted forms of microbial β-glucuronidase. Such β-glucuronidase genes may be isolated by a variety of methods, including genetic, biochemical, or immunological procedures. As exemplified herein, a gene from a *Bacillus* encoding a secreted β-glucuronidase was identified biochemically and by DNA sequence analysis. Secreted microbial β-glucuronidases from other organisms may be identified biochemically as described herein or by hybridization of the *Bacillus* β-glucuronidase gene sequence with genomic or cDNA libraries, by genetic complementation, by function, or by antibody screening of an expression library (*see* Sambrook et al., *infra* Ausubel et al, *infra* for methods and conditions appropriate for isolation of a β-glucuronidase from other species). Merely as an example, the isolation of *Bacillus* β-glucuronidase gene and gene products are provided herein.

The existence of a secreted form of β-glucuronidase may be observed by biochemical screening of samples containing microbes, such as those isolated from soil, animal or human skin, saliva, mucous, or feces, water, and the like. Colonies are plated, and a glucuronide substrate is added that is readily detectable when cleaved by β-

glucuronidase. A microbe that secretes β-glucuronidase will exhibit a diffuse staining pattern surrounding the colony. A complementation assay may be performed to verify that the staining pattern is due to a secreted GUS. In this assay, the candidate secreted GUS gene is transfected into an *E. coli* strain that is deleted for the GUS operon (*e.g.*, KW1 described herein), and the staining pattern of the transfectant is compared to a mock-transfected host. The transfectant should exhibit a diffuse staining pattern surrounding the colony, whereas, the host will not.

In an exemplary screen, a bacterial colony isolated from a soil sample displayed a strong, diffuse staining pattern. The bacterium was identified as a *Bacillus* by sequence determination of 16S rRNA after amplification. A genomic library from this *Bacillus* was constructed in the vector pBSII KS+. The recombinant plasmids were transfected into KW1, a strain deleted for the β-glucuronidase operon. One resulting colony, pRAJa17.1, exhibited a strong, diffuse staining pattern similar to the *Bacillus*.

The DNA sequence of the insert of pRAJa17.1 is presented in Figure 1 and as SEQ ID No: ___. A schematic of the insert is presented in Figure 2. The β-glucuronidase gene contained in the insert was identified by similarity of the predicted amino acid sequence of an open reading frame (Figure 3; SEQ ID No: ___) to the *E. coli* and human β-glucuronidase amino acid sequences (Figure 4). Overall, *Bacillus* β-glucuronidase has approximately 47-49% amino acid identity to *E. coli* GUS and to human GUS. An open reading frame of *Bacillus* GUS is 1854 bases, which would result in a protein that is 618 amino acids in length. The first methionine codon , however, is unlikely to encode the initiator methionine. Rather the second methionine codon, at codon 16, is most likely the initiator methionine. Such a translated product is 602 amino acids long and is the sequence presented in Figures 2 and 3. The assignment of the initiator methionine is based upon a consensus Shine-Dalgarno sequence found upstream of the second Met, but not the first Met, and alignment of the *Bacillus*, human and *E. coli* GUS amino acid sequences. Furthermore, as shown herein, *Bacillus* GUS gene lacking sequence encoding the 16 amino acids is expressed in *E. coli* transfectants. In addition, the 16 amino acids (Met-Leu-Ile-Ile-Thr-Cys-Asn-His-Leu-His-Leu-Lys-Arg-Ser-Ala-Ile) do not exhibit a consensus signal peptide sequence.

There is a single Asn-Asn-Ser sequence (residues 128-130 in Figure 3) that can serve as a site for N-linked carbohydrates. Furthermore, unlike the *E. coli* and human β-glucuronidases, which have 9 and 4 cysteines respectively, the *Bacillus* protein has only a single Cys residue (residue 499 in Figure 3).

The *Bacillus* β-glucuronidase is secreted in *E. coli* when introduced in an expression plasmid as evidenced by approximately half of the enzyme activity being detected in the periplasm. In contrast, less than 10% of *E. coli* β-glucuronidase is found in periplasm. Secreted microbial GUS is also more stable than *E. coli* GUS (Figure 7), has a higher turnover number at both 37°C and room temperature (Figures 8 and 9), and unlike *E. coli* GUS, it is not substantially inhibited by detergents (Figure 10) or by glucuronic acid (Figure 11) and retains activity in high salt conditions and organic solvents (Figure 12).

In certain aspects, variants of secreted microbial GUS are useful within the context of this invention. Variants include nucleotide or amino acid substitutions, deletions, insertions, and chimeras. Typically, when the result of synthesis, amino acid substitutions are conservative, *i.e.*, substitution of amino acids within groups of polar, non-polar, aromatic, charged, etc. amino acids. As will be appreciated by those skilled in the art, a nucleotide sequence encoding microbial GUS may differ from the wild-type sequence presented in the Figures; due to codon degeneracies, nucleotide polymorphisms, or amino acid differences. In certain embodiments, variants preferably hybridize to the wild-type nucleotide sequence at conditions of normal stringency, which is approximately 25-30°C below Tm of the native duplex (*e.g.*, 1 M Na+ at 65°C; e.g. 5X SSPE, 0.5% SDS, 5X Denhardt's solution, at 65°C or equivalent conditions; *see generally*, Sambrook et al. *Molecular Cloning: A Laboratory Manual*, 2nd ed.. Cold Spring Harbor Press, 1987; Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publishing, 1987). Alternatively, the Tm for other than short oligonucleotides can be calculated by the formula Tm=81.5 + 0.41%(G+C) - log(Na+). Low stringency hybridizations are performed at conditions approximately 40°C below Tm, and high stringency hybridizations are performed at conditions approximately 10°C below Tm.

Variants may be constructed by any of the well known methods in the art

(*see, generally,* Ausubel et al., *supra*; Sambrook et al., *supra*). Such methods include site-directed oligonucleotide mutagenesis, restriction enzyme digestion and removal or insertion of bases, amplification using primers containing mismatches or additional nucleotides, splicing of another gene sequence to the reference microbial GUS gene, and the like. Briefly, preferred methods for generating a few nucleotide substitutions utilize an oligonucleotide that spans the base or bases to be mutated and contains the mutated base or bases. The oligonucleotide is hybridized to complementary single stranded nucleic acid and second strand synthesis is primed from the oligonucleotide. Similarly, deletions and/or insertions may be constructed by any of a variety of known methods. For example, the gene can be digested with restriction enzymes and religated such that some sequence is deleted or ligated with an isolated fragment having cohesive ends so that an insertion or large substitution is made. In another embodiment. variants are generated by shuffling of regions (see U.S. Patent No. 5,605,793). Variant sequences may also be generated by "molecular evolution" techniques (see U. S. Patent No. 5,723,323). Other means to generate variant sequences may be found, for example, in Sambrook et al. (*supra*) and Ausubel et al. (*supra*). Verification of variant sequences is typically accomplished by restriction enzyme mapping, sequence analysis. or probe hybridization, although other methods may be used. The double-stranded nucleic acid is transformed into host cells, typically *E. coli*, but alternatively, other prokaryotes. yeast, or larger eukaryotes may be used. Standard screening protocols, such as nucleic acid hybridization, amplification, and DNA sequence analysis. will identify mutant sequences.

In addition to directed mutagenesis in which one or a few amino acids are altered, variants that have multiple substitutions may be generated. The substitutions may be scattered throughout the protein or functional domain or concentrated in a small region. For example, a region may be mutagenized by oligonucleotide-directed mutagenesis in which the oligonucleotide contains a string of $\bar{d}N$ bases or the region is excised and replaced by a string of dN bases. Thus, a population of variants with a randomized amino acid sequence in a region is generated.

The variant with the desired properties (*e.g.*, more efficient secretion) is then selected from the population.

As shown herein, multiple mutations at residues Val 128, Leu 141, Tyr 204 and Thr 560 (Figure 3) result in a non-functional enzyme. Thus, at least one of these amino acids is critical to maintaining enzyme activity. A mutein *Bacillus* GUS containing the amino acid alterations of Val 128 →Ala, Leu 141→His, Tyr 204→Asp and Thr 560→Ala was constructed and exhibited little enzymatic activity. As shown herein, the residue alteration that most directly affected activity is Leu 141. In addition, three residues have been identified as likely contact residues important for catalysis in human GUS (residues Glu 451, Glu 540, and Tyr 504) (Jain et al., *Nature Struct. Biol.* 3: 375, 1996). Based on alignment with *Bacillus* GUS, the corresponding residues are Glu 415, Glu 508, and Tyr 471. By analogy with human GUS, Asp 165 may also be close to the reaction center and likely forms a salt bridge with Arg 566. Thus, in embodiments where it is desirable to retain enzymatic activity of GUS, the residues corresponding Leu 141, Glu 415, Glu 508, Tyr 471, Asp 165, and Arg 566 in *Bacillus* GUS are preferably unaltered.

In preferred embodiments, the protein and variants are capable of being secreted and exhibit β-glucuronidase activity. In other preferred embodiments, one or more of the biochemical characteristics exhibited by *Bacillus* GUS, such as its increased stability, its higher turnover number, and its activity in detergents, presence of end product, high salt conditions and organic solvents as compared to *Ec*GUS, are retained in GUS and variants thereof. In other preferred embodiments, GUS and variants thereof are capable of being secreted and exhibit one or more of the biochemical characteristics disclosed herein. In other embodiments, variants of microbial GUS are capable of binding to a hapten, such as biotin, dinitrophenol, and the like.

In other embodiments, variants may exhibit glucuronide binding activity without enzymatic activity or be directed to other cellular compartments, such as membrane or cytoplasm. Membrane-spanning amino acid sequences are generally hydrophobic and many examples of such sequences are well-known. These sequences may be spliced onto microbial secreted GUS by a variety of methods including

conventional recombinant DNA techniques. Similarly, sequences that direct proteins to cytoplasm (*e.g.*, Lys-Asp-Glu-Leu) may be added to the reference GUS, typically by recombinant DNA techniques.

In other embodiments, the nucleic acid molecule encoding microbial GUS may be fused to another nucleic acid molecule. As will be appreciated, the fusion partner gene may contribute, within certain embodiments, a coding region. In a preferred embodiment, microbial GUS is fused to avidin or streptavidin. Thus, it may be desirable to use only the catalytic site of GUS (*e.g.*, amino acids 415-508). The choice of the fusion partner depends in part upon the desired application. The fusion partner may be used to alter specificity of GUS, provide a reporter function, provide a tag sequence for identification or purification protocols, and the like. The reporter or tag can be any protein that allows convenient and sensitive measurement or facilitates isolation of the gene product and does not interfere with the function of GUS. For example, green fluorescent protein and β-galactosidase are readily available as DNA sequences. A peptide tag is a short sequence, usually derived from a native protein, which is recognized by an antibody or other molecule. Peptide tags include FLAG®, Glu-Glu tag (Chiron Corp., Emeryville, CA) KT3 tag (Chiron Corp.), T7 gene 10 tag (Invitrogen, La Jolla, CA), T7 major capsid protein tag (Novagen, Madison, WI), His_6 (hexa-His), and HSV tag (Novagen). Besides tags, other types of proteins or peptides, such as glutathione-S-transferase may be used.

In addition, portions or fragments of microbial GUS may be isolated or constructed for use in the present invention. For example, restriction fragments can be isolated by well-known techniques from template DNA, *e.g.*, plasmid DNA, and DNA fragments, including restriction fragments, can be generated by amplification. Furthermore, oligonucleotides can be synthesized or isolated from recombinant DNA molecules. One skilled in the art will appreciated that other methods are available to obtain DNA or RNA molecules having at least a portion of a microbial GUS sequence. Moreover, for particular applications, these nucleic acids may be labeled by techniques known in the art with a radiolabel (*e.g.*, ^{32}P, ^{33}P, ^{35}S, ^{125}I ^{131}I, ^{3}H, ^{14}C). fluorescent label (*e.g.*, FITC, Cy5, RITC, Texas Red), chemiluminescent label, enzyme, biotin and the

like.

In other aspects of the present invention, isolated microbial glucuronidase proteins are provided. In one embodiment, GUS protein is expressed as a hexa-his fusion protein and isolated by metal-containing chromatography, such as nickel-coupled beads. Briefly, a sequence encoding His_6 is linked to a DNA sequence encoding a GUS. Although the His_6 sequence can be positioned anywhere in the molecule, preferably it is linked at the 3' end immediately preceding the termination codon. The His-GUS fusion may be constructed by any of a variety of methods. A convenient method is amplification of the GUS gene using a downstream primer that contains the codons for His_6.

In one aspect of the present invention, peptides having microbial GUS sequence are provided. Peptides may be used as immunogens to raise antibodies, as well as other uses. Peptides are generally five to 100 amino acids long, and more usually 10 to 50 amino acids. Peptides are readily chemically synthesized in an automated fashion (PerkinElmer ABI Peptide Synthesizer) or may be obtained commercially. Peptides may be further purified by a variety of methods, including high-performance liquid chromatography. Furthermore, peptides and proteins may contain amino acids other than the 20 naturally occurring amino acids or may contain derivatives and modification of the amino acids.

β-glucuronidase protein may be isolated by standard methods, such as affinity chromatography using matrices containing saccharose lactone, phenylthio-β-glucuronide, antibodies to GUS protein and the like, size exclusion chromatography, ionic exchange chromatography, HPLC, and other known protein isolation methods. (*see generally* Ausubel et al. *supra*; Sambrook et al. *supra*). The protein can be expressed as a hexa-His fusion protein and isolated by metal-containing chromatography, such as nickel-coupled beads. An isolated purified protein gives a single band on SDS-PAGE when stained with Coomassie blue.

Antibodies to microbial GUS

Antibodies to microbial GUS proteins, fragments, or peptides discussed herein may readily be prepared. Such antibodies may specifically recognize reference

microbial GUS protein and not a mutant (or variant) protein, mutant (or variant) protein and not wild type protein, or equally recognize both the mutant (or variant) and wild-type forms. Antibodies may be used for isolation of the protein, inhibiting (antagonist) activity of the protein, or enhancing (agonist) activity of the protein.

Within the context of the present invention, antibodies are understood to include monoclonal antibodies, polyclonal antibodies, anti-idiotypic antibodies, antibody fragments (*e.g.*, Fab, and $F(ab')_2$, F_V variable regions, or complementarity determining regions). Antibodies are generally accepted as specific against GUS protein if they bind with a K_d of greater than or equal to 10^{-7}M, preferably greater than of equal to 10^{-8}M. The affinity of a monoclonal antibody or binding partner can be readily determined by one of ordinary skill in the art (*see* Scatchard, *Ann. N.Y. Acad. Sci. 51*:660-672, 1949).

Briefly, a polyclonal antibody preparation may be readily generated in a variety of warm-blooded animals such as rabbits, mice, or rats. Typically, an animal is immunized with GUS protein or peptide thereof, which may be conjugated to a carrier protein, such as keyhole limpet hemocyanin. Routes of administration include intraperitoneal, intramuscular, intraocular, or subcutaneous injections, usually in an adjuvant (*e.g.*, Freund's complete or incomplete adjuvant). Particularly preferred polyclonal antisera demonstrate binding in an assay that is at least three times greater than background.

Monoclonal antibodies may also be readily generated from hybridoma cell lines using conventional techniques (*see* U.S. Patent Nos. RE 32,011, 4,902,614, 4,543,439, and 4,411,993; *see also Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988). Briefly, within one embodiment, a subject animal such as a rat or mouse is injected with GUS or a portion thereof. The protein may be administered as an emulsion in an adjuvant such as Freund's complete or incomplete adjuvant in order to increase the immune response. Between one and three weeks after the initial immunization the animal is generally boosted and may tested for reactivity to the protein utilizing well-known assays. The spleen and/or lymph nodes are harvested and immortalized. Various immortalization techniques, such as mediated

by Epstein-Barr virus or fusion to produce a hybridoma, may be used. In a preferred embodiment, immortalization occurs by fusion with a suitable myeloma cell line (e.g., NS-1 (ATCC No. TIB 18), and P3X63 - Ag 8.653 (ATCC No. CRL 1580) to create a hybridoma that secretes monoclonal antibody. The preferred fusion partners do not express endogenous antibody genes. Following fusion, the cells are cultured in medium containing a reagent that selectively allows for the growth of fused spleen and myeloma cells such as HAT (hypoxanthine, aminopterin, and thymidine) and are subsequently screened for the presence of antibodies that are reactive against a GUS protein. A wide variety of assays may be utilized, including for example countercurrent immuno-electrophoresis, radioimmunoassays, radioimmunoprecipitations, enzyme-linked immunosorbent assays (ELISA), dot blot assays, western blots, immunoprecipitation, inhibition or competition assays, and sandwich assays (*see* U.S. Patent Nos. 4.376,110 and 4,486,530; *see also Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988).

Other techniques may also be utilized to construct monoclonal antibodies (*see* Huse et al., *Science 246*:1275-1281, 1989; Sastry et al., *Proc. Natl. Acad. Sci. USA 86*:5728-5732, 1989; Alting-Mees et al., *Strategies in Molecular Biology 3*:1-9. 1990; describing recombinant techniques). Briefly, RNA is isolated from a B cell population and utilized to create heavy and light chain immunoglobulin cDNA expression libraries in suitable vectors, such as λImmunoZap(H) and λImmunoZap(L). These vectors may be screened individually or co-expressed to form Fab fragments or antibodies (*see* Huse et al., *supra*; Sastry et al., *supra*). Positive plaques may subsequently be converted to a non-lytic plasmid that allows high level expression of monoclonal antibody fragments from *E. coli*.

Similarly, portions or fragments, such as Fab and Fv fragments, of antibodies may also be constructed utilizing conventional enzymatic digestion or recombinant DNA techniques to yield isolated variable regions of an antibody. Within one embodiment, the genes which encode the variable region from a hybridoma producing a monoclonal antibody of interest are amplified using nucleotide primers for the variable region. These primers may be synthesized by one of ordinary skill in the

art, or may be purchased from commercially available sources (*e.g.*, Stratacyte, La Jolla, CA) Amplification products are inserted into vectors such as ImmunoZAP™ H or ImmunoZAP™ L (Stratacyte), which are then introduced into *E. coli*, yeast, or mammalian-based systems for expression. Utilizing these techniques, large amounts of a single-chain protein containing a fusion of the V_H and V_L domains may be produced (*see* Bird et al., *Science 242*:423-426, 1988). In addition, techniques may be utilized to change a "murine" antibody to a "human" antibody, without altering the binding specificity of the antibody.

Once suitable antibodies have been obtained, they may be isolated or purified by many techniques well known to those of ordinary skill in the art (*see Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988). Suitable techniques include peptide or protein affinity columns, HPLC or RP-HPLC, purification on protein A or protein G columns, or any combination of these techniques.

Assays for function of β-glucuronidase

In preferred embodiments, microbial β-glucuronidase will have at least enzymatic activity and capability of being secreted. As noted above, variants of these reference GUS proteins may exhibit altered functional activity and cellular localization. Enzymatic activity may be assessed by an assay such as the ones disclosed herein or in U.S. Patent No. 5,268,463 (Jefferson). Generally, a chromogenic or fluorogenic substrate is incubated with cell extracts, tissue sections, or purified protein. Cleavage of the substrate is monitored by a method appropriate for the aglycon.

A variety of methods may be used to demonstrate that a β-glucuronidase is secreted. For example, a rapid screening method in which colonies of organisms or cells, such as bacteria, yeast or insect cells, are plated and incubated with a readily visualized glucuronide substrate, such as X-glcA. A colony with a diffuse staining pattern likely secretes GUS, although such a pattern could indicate that the cell has the ability to pump out the cleaved glucuronide or that the enzyme is membrane bound. When test cells express GUS from an introduced vector, a cell that is known to not pump out cleaved substrate is preferably used.

Secretion of the enzyme may be verified by assaying for GUS activity in the extracellular environment. If the cells secreting GUS are gram-positive bacteria, yeasts, molds, plants, or other organisms with cell walls, activity may be assayed in the culture medium and in a cell extract, however, the protein may not be transported through the cell wall. Thus, if no or low activity of a secreted form of GUS is found in the culture medium, protoplasts can be made by osmotic shock or enzymatic digestion of the cell wall or other suitable procedure, and the supernatant assayed for GUS activity. If the cells secreting GUS are gram-negative bacteria, culture supernatant may be tested, but more likely β-glucuronidase will be retained in the periplasmic space between the inner and outer membrane. In this case, spheroplasts may be made by osmotic shock, enzymatic digestion, or other suitable procedure, and the supernatant assayed for GUS activity. Other cells, without cell walls, are assayed for GUS in cell supernatant and cell extracts. The fraction of activity in each compartment is compared to the activity of a non-secreted GUS in the same or similar host cells. A β-glucuronidase is secreted if significantly more enzyme activity than *E. coli* GUS activity is found in extracellular spaces. Less than 10% of *E. coli* GUS is secreted. Higher amounts of secreted enzyme are preferred (*e.g.*, greater than 20%, 25%, 30%, 40%, 50%).

Vectors, host cells and means of expressing and producing protein

Microbial β-glucuronidase may be expressed in a variety of host organisms. For protein production and purification, secreted GUS is preferably produced in bacteria, such as *E. coli*, for which many expression vectors have been developed and are available. Other suitable host organisms include other bacterial species (e.g., *Bacillus*, and eukaryotes, such as yeast (*e.g.*, *Saccharomyces cerevisiae*), mammalian cells (*e.g.*, CHO and COS-7), plant cells and insect cells (*e.g.*, Sf9). Vectors for these hosts are well known.

A DNA sequence encoding a secreted form of β-glucuronidase is introduced into an expression vector appropriate for the host. The sequence is derived from an existing clone or synthesized. A preferred means of synthesis is amplification

of the gene from cDNA, genomic DNA, or a recombinant clone using a set of primers that flank the coding region or the desired portion of the protein. Restriction sites are typically incorporated into the primer sequences and are chosen with regard to the cloning site of the vector. If necessary, translational initiation and termination codons can be engineered into the primer sequences. The sequence of GUS can be codon-optimized for expression in a particular host. For example, a secreted form of β-glucuronidase isolated from a bacterial species that is expressed in a fungal host, such as yeast, is altered in nucleotide sequence to use codons preferred in yeast. Codon-optimization may be accomplished by methods such as splice overlap extension, site-directed mutagenesis, automated synthesis, and the like.

At minimum, the vector must contain a promoter sequence. Other regulatory sequences may be included. Such sequences include a transcription termination signal sequence, secretion signal sequence, origin of replication, selectable marker, and the like. The regulatory sequences are operationally associated with one another to allow transcription or translation.

Expression in bacteria

The plasmids used herein for expression of secreted GUS include a promoter designed for expression of the proteins in a bacterial host. Suitable promoters are widely available and are well known in the art. Inducible or constitutive promoters are preferred. Such promoters for expression in bacteria include promoters from the T7 phage and other phages, such as T3, T5, and SP6, and the trp, lpp, and lac operons. Hybrid promoters (*see*, U.S. Patent No. 4,551,433), such as tac and trc, may also be used. Promoters for expression in eukaryotic cells include the P10 or polyhedron gene promoter of baculovirus/insect cell expression systems (*see*, *e.g.*, U.S. Patent Nos. 5,243,041, 5,242,687, 5,266,317, 4,745,051, and 5,169,784), MMTV LTR, RSV LTR, SV40, metallothionein promoter (*see*, *e.g.*, U.S. Patent No. 4,870,009) and other inducible promoters. For expression of the proteins, a promoter is inserted in operative linkage with the coding region for β-glucuronidase.

The promoter controlling transcription of β-glucuronidase may be controlled by a repressor. In some systems, the promoter can be derepressed by altering

the physiological conditions of the cell, for example, by the addition of a molecule that competitively binds the repressor, or by altering the temperature of the growth media. Preferred repressor proteins include, but are not limited to the *E. coli* lacI repressor responsive to IPTG induction, the temperature sensitive λcI857 repressor, and the like. The *E. coli* lacI repressor is preferred.

In other preferred embodiments, the vector also includes a transcription terminator sequence. A "transcription terminator region" has either a sequence that provides a signal that terminates transcription by the polymerase that recognizes the selected promoter and/or a signal sequence for polyadenylation.

Preferably, the vector is capable of replication in bacterial cells. Thus, the vector preferably contains a bacterial origin of replication. Preferred bacterial origins of replication include the f1-ori and col E1 origins of replication, especially the ori derived from pUC plasmids.

The plasmids also preferably include at least one selectable marker that is functional in the host. A selectable marker gene includes any gene that confers a phenotype on the host that allows transformed cells to be identified and selectively grown. Suitable selectable marker genes for bacterial hosts include the ampicillin resistance gene (Amp^r), tetracycline resistance gene (Tc^r) and the kanamycin resistance gene (Kan^r). The kanamycin resistance gene is presently preferred. Suitable markers for eukaryotes usually require a complementary deficiency in the host (*e.g.*, thymidine kinase (tk) in tk- hosts). However, drug markers are also available (*e.g.*. G418 resistance and hygromycin resistance).

The sequence of nucleotides encoding β-glucuronidase may also include a classical secretion signal, whereby the resulting peptide is a precursor protein processed and secreted. The resulting processed protein may be recovered from the periplasmic space or the fermentation medium. Secretion signals suitable for use are widely available and are well known in the art (von Heijne, *J. Mol. Biol. 184*:99-105, 1985). Prokaryotic and eukaryotic secretion signals that are functional in *E. coli* (or other host) may be employed. The presently preferred secretion signals include. but are not limited to, those encoded by the following *E. coli* genes: pelB (Lei et al., *J.*

Bacteriol. 169:4379, 1987), phoA, ompA, ompT, ompF, ompC, beta-lactamase, and alkaline phosphatase.

One skilled in the art appreciates that there are a wide variety of suitable vectors for expression in bacterial cells and which are readily obtainable. Vectors such as the pET series (Novagen, Madison, WI) and the tac and trc series (Pharmacia, Uppsala, Sweden) are suitable for expression of a β-glucuronidase. A preferred vector is the backbone of pLAD-F48 (Figure 15). This plasmid is ampicillin resistant, has a colEI origin of replication, $lacI^q$ gene, a lac/trp hybrid promoter in front of the lac Shine-Dalgarno sequence, a hexa-his coding sequence that joins to the 3' end of the inserted gene, and an rrnB terminator sequence.

The choice of a bacterial host for the expression of a β-glucuronidase is dictated in part by the vector. Commercially available vectors are paired with suitable hosts. The vector is introduced in bacterial cells by standard methodology. Typically, bacterial cells are treated to allow uptake of DNA (for protocols, see generally, Ausubel et al., *supra*; Sambrook et al., *supra*). Alternatively, the vector may be introduced by electroporation, phage infection, or another suitable method.

Expression in plant cells

As noted above, the present invention provides vectors capable of expressing secreted β-glucuronidase. For agricultural applications, the vectors should be functional in plant cells. Vectors and procedures for cloning and expression in *E. coli* and animal cells are discussed herein and, for example, in Sambrook et al (*supra*) and in Ausubel et al. (*supra*). In one embodiment, rice is a host for GUS gene expression.

Vectors that are functional in plants are preferably binary plasmids derived from *Agrobacterium* plasmids. Such vectors are capable of transforming plant cells. These vectors contain left and right border sequences that are required for integration into the host (plant) chromosome. At minimum, between these border sequences is the gene to be expressed under control of a promoter. In preferred embodiments, a selectable marker and a reporter gene are also included. The vector also preferably contains a bacterial origin of replication.

A gene for microbial β-glucuronidase should be in operative linkage with a promoter. The promoter should be functional in a plant cell. Typically, the promoter is derived from a host plant gene, but promoters from other plant species and other organisms, such as insects, fungi, viruses, mammals, and the like, may also be suitable, and at times preferred. The promoter may be constitutive or inducible, or may be active in a certain tissue or tissues (tissue type-specific promoter), in a certain cell or cells (cell-type specific promoter), of at a particular stage or stages of development (development-type specific promoter). The choice of a promoter depends at least in part upon the application. Many promoters have been identified and isolated (*see, generally*, GenBank and EMBL databases). Other promoters may be isolated by well-known methods. For example, a genomic clone for a particular gene can be isolated by probe hybridization. The coding region is mapped by restriction mapping, DNA sequence analysis, RNase probe protection, or other suitable method. The genomic region immediately upstream of the coding region comprises a promoter region and is isolated. Generally, the promoter region is located in the first 200 bases upstream, but may extend to 500 or more bases. The candidate region is inserted in a suitable vector in operative linkage with a reporter gene, such as in pBI121 in place of the CaMV 35S promoter, and the promoter is tested by assaying for the reporter gene after transformation into a plant cell. (*see, generally,* Ausubel et al., *supra*; Sambrook et al., *supra*; *Methods in Plant Molecular Biolgoy and Biotechnology*, Ed. Glick and Thompson, CRC Press, 1993.)

Preferably, the vector contains a selectable marker for identifying transformants. The selectable marker preferably confers a growth advantage under appropriate conditions. Generally, selectable markers are drug resistance genes, such as neomycin phosphotransferase. Other drug resistance genes are known to those in the art and may be readily substituted. The selectable marker also preferably has a linked constitutive or inducible promoter and a termination sequence, including a polyadenylation signal sequence.

Additionally, a bacterial origin of replication and a selectable marker for bacteria are preferably included in the vector. Of the various origins (*e.g.*, colEI, fd

phage), a colEI origin of replication is preferred. Most preferred is the origin from the pUC plasmids, which allow high copy number. Selectable markers for bacteria include, ampicillin resistance, tetracycline resistance, kanamycin resistance, chloramphenicol resistance, and the like.

The sequence of nucleotides encoding β-glucuronidase may also include a classical secretion signal, whereby the resulting peptide is a precursor protein processed and secreted. Suitable signal sequences of plant genes include, but are not limited to the signal sequences from glycine-rich protein and extensin. In addition, a glucuronide permease gene may be co-transfected either from the same vector containing microbial GUS or from a separate expression vector.

A general vector suitable for use in the present invention is based on pBI121 (U.S. Patent No. 5,432,081) a derivative of pBIN19. Other vectors have been described (U.S. Patent No. 4,536,475) or may be constructed based on the guidelines presented herein. The plasmid pBI121 contains a left and right border sequence for integration into a plant host chromosome and also contains a bacterial origin of replication and selectable marker. These border sequences flank two genes. One is a kanamycin resistance gene (neomycin phosphotransferase) driven by a nopaline synthase promoter and using a nopaline synthase polyadenylation site. The second is the *E. coli* GUS gene (reporter gene) under control of the CaMV 35S promoter and polyadenlyated using a nopaline synthase polyadenylation site. The *E. coli* GUS gene is replaced with a gene encoding a secreted form of β-glucuronidase. If appropriate, the CaMV 35S promoter is replaced by a different promoter. Either one of the expression units described above is additionally inserted or is inserted in place of the CaMV promoter and GUS gene.

Plants may be transformed by any of several methods. For example, plasmid DNA may be introduced by *Agrobacterium* co-cultivation or bombardment. Other transformation methods include electroporation, $CaPO_4$-mediated transfection, gene transfer to protoplasts, microinjection, and the like (*see, Gene Transfer to Plants*, Ed. Potrykus and Spangenberg, Springer, 1995, for procedures). Preferably, vector DNA is first transfected into *Agrobacterium* and subsequently introduced into plant

cells. Most preferably, the infection is achieved by co-cultivation. In part, the choice of transformation methods depends upon the plant to be transformed. For example, monocots generally cannot be transformed by *Agrobacterium*. Thus, *Agrobacterium* transformation by co-cultivation is most appropriate for dicots and for mitotically active tissue. Non-mitotic dicot tissues can be efficiently infected by *Agrobacterium* when a projectile or bombardment method is utilized. Projectile methods are also generally used for transforming sunflowers and soybean. Bombardment is used when naked DNA, typically *Agrobacterium* or pUC-based plasmids, is used for transformation or transient expression.

Briefly, co-cultivation is performed by first transforming *Agrobacterium* by freeze-thawing (Holsters et al., *Mol. Gen. Genet. 163*: 181-187, 1978) or by other suitable methods (*see,* Ausubel, et al. *supra*; Sambrook et al., *supra*). A culture of *Agrobacterium* containing the plasmid is incubated with leaf disks, protoplasts or meristematic tissue to generate transformed plants (Bevan, *Nucl. Acids. Res. 12*:8711, 1984).

Briefly, for microprojectile bombardment, seeds are surface sterilized in bleach solution and rinsed with distilled water. Seeds are then imbibed in distilled water, and the cotyledons are broken off to produce a clean fracture at the plane of the embryonic axis. Explants are then bisected longitudinally between the primordial leaves and placed cut surface up on medium with growth regulating hormones. minerals and vitamin additives. Explants are bombarded with 1.8 μm tungsten microprojectiles by a particle acceleration device. Freshly bombarded explants are placed in a suspension of transformed *Agrobacterium* transferred to medium with the cut surfaces down for 3 days with an 18 hr light cycle. Explants are transferred to medium lacking growth regulators but containing drug for selection and grown for 2-5 weeks. After 1-2 weeks more without drug selection, leaf samples from green, drug-resistant shoots are grafted to in vitro grown rootstock and transferred to soil.

Activity of secreted GUS is assayed in whole plants or in selected tissues using a glucuronide substrate that is readily detected upon cleavage. Glucuronide substrates that are colorimetric are preferred. Field testing of plants may be performed

by spraying a plant with the glucuronide substrate and observing color formation of the cleaved product.

Expression in other organisms

A variety of other organisms are suitable for use in the present invention. For example, various fungi, including yeasts, molds, and mushrooms, insects, especially vectors for diseases and pathogens, and other animals, such as cows, mice, goats, and the like, may be transformed with a GUS transgene.

The principles that guide vector construction for bacteria and plants, as discussed above, are applicable to vectors for these organisms. In general, vectors are well known and readily available. Briefly, the vector should have a promoter in operative linkage with GUS. Usually, the vector will also have one or more selectable markers, an origin of replication, a polyadenylation signal and transcription terminator.

The sequence of nucleotides encoding β-glucuronidase may also include a classical secretion signal, whereby the resulting peptide is a precursor protein processed and secreted. Suitable secretion signals may be obtained from mat-alpha or invertase genes for example. In addition, a permease gene may be co-transfected.

Uses of microbial β-glucuronidase

As noted above, microbial β-glucuronidase may be used in a variety of applications. In general, microbial β-glucuronidase can be used as a reporter/effector molecule and as a diagnostic tool. As taught herein, microbial β-glucuronidase that is secretable is advantageous as a reporter/effector molecule, whereas, in dignostic applications, the biochemical characteristics of the β-glucuronidase disclosed herein provide advantages.

Secreted microbial GUS can be used as a marker for transgenic constructions. In a preferred embodiment, the transgenic host is a plant, such as rice, corn, wheat. The transgenic GUS may be used in at least two ways: one in a method of positive selection, obviating the need for drug resistance selection, and a second as a means of detecting and tracking linked genes.

For positive selection, the plant cell is transformed with a s-GUS (secretable GUS) transgene. Selection is achieved by providing the cells with a

gluronidated form of a required nutrient. For example, all cells require a carbon source, such as glucose. In one embodiment, glucose is provided as glucuronyl glucose, which is cleaved by s-GUS into glucose plus glucuronic acid. The glucose would then bind to receptors and be taken up by cells. The glucuronide may be any required compound, including without limitation, a cytokinin, auxin, vitamin, carbohydrate, nitrogen-containing compound, and the like. It will be appreciated that this positive selection method can be used for cells and tissues derived from diverse organisms, such as animal cells, insect cells, fungi, and the like. The choice of glucuronide will depend in part upon the requirements of the host cell.

As a marker, s-GUS is preferred because it is non-destructive, that is, the host does not need to be destroyed in order to assay enzyme activity. A non-destructive marker has special utility as a tool in plant breeding. The GUS enzyme can be used to detect and track linked endogenous or exogenously introduced genes. s-GUS may also be used to generate sentinel plants that serve as bioindicators of environmental status. Plant pathogen invasion can be monitored if GUS is under control of a pathogen promoter. In addition, such transgenic plants may serve as a model system for screening inhibitors of pathogen invasion. In this system, GUS is expressed if a pathogen invades. In the presence of an effective inhibitor, GUS activity will not be detectable. In certain embodiments, s-GUS is co-transfected with a gene encoding a glucuronide permease.

Preferred transgenes for introduction into plants encode proteins that affect fertility, including male sterility, female fecundity, and apomixis; plant protection genes, including proteins that confer resistance to diseases, bacteria, fungus, nemotodes, viruses and insects; genes and proteins that affect developmental processes or confer new phenotypes, such as genes that control development of meristem, timing of flowering, and the such.

Insect and disease resistance genes are well known. Some of these genes are present in the genome of plants and have been genetically identified. Others of these genes have been found in bacteria and are used to confer resistance.

Particularly well known insect resistance genes are the crystal genes of *Bacillus thuringiensis*. The crystal genes are active against various insects, such as lepidopterans, *Diptera*, and mosquitos. Many of these genes have been cloned. For examples, see, GenBank Accession Nos. X96682, X96684; M76442, M90843, M89794, M22472, M37207, D17518, L32019, M97880, L32020, M64478, M11250, M13201, D00117, M73319, X17123, X86902, X06711, X13535, X54939, X54159, X13233, X54160, X56144, X58534, X59797, X75019, X62821, Z46442, U07642, U35780, U43605, U43606, U10985; U.S. Patent Nos. 5,317,096; 5,254,799; 5,460,963; 5,308,760, 5,466,597, 5,2187,091, 5,382,429, 5,164,180, 5,206,166, 5,407,825, 4,918,066; PCT Applications WO 95/30753, WO 94/24264; AU 9062083; EP 408403 B1, EP 142924 B1, EP 256,553 B1, EP 192,741 B1; JP 62-56932; . Gene sequences for these and related proteins may be obtained by standard and routine technologies, such as probe hybridization of a *B. thuringiensis* library or amplification (*see generally*, Sambrook et al., *supra*, Ausubel et al. *supra*). The probes and primers may be synthesized based on publicly available sequence information.

Other resistance genes to *Sclerotinia*, cyst nematodes, tobacco mosaic virus, flax and crown rust, rice blast, powdery mildew, verticillum wilt, potato beetle, aphids, as well as other infections, are useful within the context of this invention. Examples of such disease resistance genes may be isolated from teachings in the following references: isolation of rust disease resistance gene from flax plants (WO 95/29238); isolation of the gene encoding Rps2 protein from *Arabidopsis thaliana* that confers disease resistance to pathogens carrying the avrRpt2 avirulence gene (WO 95/28478); isolation of a gene encoding a lectin-like protein of kidney bean confers insect resistance (JP 71-32092); isolation of the Hm1 disease resistance gene to *C. carbonum* from maize (WO 95/07989); for examples of other resistance genes, see WO 95/05743; U.S. Patent No. 5,496,732; U.S. Patent No. 5,349,126; EP 616035; EP 392225; WO 94/18335; JP 43-20631; EP 502719; WO 90/11770; U.S. Patent 5,270,200; U.S. Patent Nos. 5,218,104 and 5,306,863). In addition, general methods for identification and isolation of plant disease resistance genes are disclosed (WO 95/28423). Any of these gene sequences suitable for insertion in a vector according to

the present invention may be obtained by standard recombinant technology techniques, such as probe hybridization or amplification. When amplification is performed, restriction sites suitable for cloning are preferably inserted. Nucleotide sequences for other transgenes, such as controlling male fertility, are found in U.S. Patent No. 5,478,369, references therein, and Mariani et al., *Nature 347*:737, 1990.

In similar fashion, secreted GUS can be used to generate transgenic insects for tracking insect populations or facilitate the development of a bioassay for compounds that affect molecules critical for insect development (*e.g.*, juvenile hormone). Secreted GUS may also serve as a marker for beneficial fungi destined for release into the environment. The non-destructive marker is useful for detecting persistance and competitive advantage of the released organisms.

In animal systems, secreted GUS may be used to achieve extracellular detoxification of glucuronides (e.g, toxin glucuronide) and examine conjugation patterns of glucuronides. Furthermore, as discussed above, secreted GUS may be used as a transgenic marker to track cells or as a positive selection system, or to assist in development of new bioactive GUS substrates that do not need to be transported across membrane.

In one aspect, microbial purified β-glucuronidase is used in medical applications. For these applications, secretion is not a necessary characteristic. The biochemical attributes, such as increased stability and enzymatic activity disclosed herein are preferred characteristics. The microbial glucuronidase preferably has one or more of the disclosed characteristics.

For the majority of drug or pharmaceutical analysis, the compounds in urine, blood, saliva, or other bodily fluids are de-glucuronidated prior to analysis. Such procedure is undertaken because compounds are often, if not nearly always, detoxified by glucuronidation. Thus, drugs that are in circulation and have passed through a site of glucuronidation (*e.g.*, liver) are found conjugated to glucuronic acid. Such glucuronides yield a complex pattern upon analysis by, for example, HPLC. However, after the aglycon (drug) is cleaved from the glucuronic acid, a spectrum can be compared to a

reference spectrum. Currently, *E. coli* GUS is utilized, but as shown herein. *Bacillus* GUS has superior qualities.

The microbial GUS enzymes disclosed herein may be used in traditional medical diagnostic assays, such as described above for drug testing, pharmacokinetic studies, bioavailability studies, diagnosis of diseases and syndromes, following progression of disease or its response to therapy and the like. These β-glucuronidase enzymes may be used in place of other traditional enzymes (*e.g.*, alkaline phosphatase, horseradish peroxidase, beta-galactosidase, and the like) and compounds (e.g., green fluorescent protein, radionuclides) that serve as visualizing agents. Microbial GUS has critical qualities for use as a visualizing agent: it is highly specific for the substrate, water soluble and the substrates are stable. Thus, microbial GUS is suitable for use in southern analysis of DNA, northern analysis, ELISA, and the like. In preferred embodiments, microbial GUS binds a hapten, either as a fusion protein with a partner protein that binds the hapten (e.g., avidin that binds biotin) or alone. If used alone, microbial GUS can be mutagenized and selected for hapten-binding abilities. Mutagenesis and binding assays are well known in the art. In addition, microbial GUS can be conjugated to avidin, streptavidin, or other hapten binding protein and used as a reporter in the myriad assays that currently employ enzyme-linked binding proteins. Such assays include immunoassays, Western blots, in situ hybridizations, HPLC, high-throughput binding assays, and the like (see, for examples, U.S. Patent Nos. 5.328.985 and 4839293, which teach avidin and streptavidin fusion proteins and U.S. Patent No. 4298685, Diamandis and Christopoulos, *Clin. Chem. 37*:625, 1991; Richards. *Methods Enzymol. 184*:3, 1990; Wilchek and Bayer, *Methods Enzymol. 184*:467, 1990; Wilchek and Bayer, *Methods Enzymol. 184*:5, 1990; Wilchek and Bayer, *Methods Enzymol. 184*:14, 1990; Dunn, *Methods Mol. Biol. 32*:227, 1994; Bloch, *J. Hitochem. Cytochem. 41*:1751, 1993; Bayer and Wilchek *J. Chromatogr. 510*:3, 1990, which teach various applications of enzyme-linked technologies and methods).

The present invention also provides kits comprising microbial GUS protein or expression vectors containing microbial GUS gene. One exemplary type of kit is a dipstick test. Such tests are widely utilized for establishing pregnancy, as well

as other conditions. Generally, these dipstick tests assay the glucuronide form. but it would be advantageous to use reagents that detect the aglycon form. Thus, GUS may be immobilized on the dipstick adjacent to or mixed in with the detector molecule (*e.g.*, antibody). The dipstick is then dipped in the test fluid (*e.g.*, urine) and as the compounds flow past GUS, they are cleaved into aglycon and glucuronic acid. The aglycon is then detected. Such a setup may be extremely useful for testing compounds that are not readily detectable as glucuronides.

In a variation of this method, the microbial GUS enzyme is engineered to bind a glucuronide but lacks enzymatic activity. The enzyme will then bind the glucuronide and the enzyme is detected by standard methodology. Alternatively, GUS is fused to a second protein, either as a fusion protein or as a chemical conjugate, that binds the aglycon. The fusion is incubated with the test substance and an indicator substrate is added. This procedure may be used for ELISA, Northern, Southern analysis and the like.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

EXAMPLE 1

ISOLATION OF A GENE ENCODING SECRETED β-GLUCURONIDASE

Soil samples are placed in broth and plated for growth of bacterial colonies on agar plates containing 50 μg/ml X-glcA (5-bromo-4-chloro-3-indolyl glucuronide), an indicator substrate for β-glucuronidase. This substrate gives a blue precipitate at the site of enzyme activity (see U.S. Patent No. 5,268,463). Bacteria that secrete β-glucuronidase have a strong, diffuse staining pattern surrounding the colony.

One bacterial colony that exhibited this type of staining pattern is chosen. The bacterium is identified as a *Bacillus* based on amplification of 16S rRNA. Oligonucleotide sequences derived from areas exhibiting a high degree of similarity between *E. coli* and human β-glucuronidases are used in amplification reactions on *Bacillus* and *E. coli* DNA. A fragment is observed using *Bacillus* DNA, which is the same size as the *E. coli* fragment.

Bacillus DNA is digested with *Hind* III and ligated to *Hind* III-digested pBSII-KS plasmid vector. The recombinant plasmid is transfected into KW1, an *E. coli* strain that is deleted for the GUS operon. Cells are plated on X-glcA plates, and one colony exhibited strong, diffuse staining pattern, suggesting that this clone encoded a secreted β-glucuronidase enzyme. The plasmid, pRAJa17.1, is isolated and subjected to analysis.

The DNA sequence of the insert of pRAJa17.1 is shown in Figure 1. A schematic of the 6029 bp fragment is shown in Figure 2. The fragment contains four large open reading frames. The open reading frame proposed as secreted GUS (*Bo*GUS) begins at nucleotide 1662 and extends to 3467 (Figure 1). The predicted translate is shown in Figure 3 and its alignment with *E. coli* and human β-glucuronidase is presented in Figure 4. *Bo*GUS is 47.2% identical to *E. coli* GUS, which is about the same identity as human GUS and *E. coli* GUS (49.1%). Thus, GUS from *Bacillus* is about as related to another bacterium as to human. One striking difference in sequence

among the proteins is the number of cysteine residues. Whereas, both human and *E. coli* GUS have 4 and 9 cysteines, respectively, *Bo*GUS has only one cysteine.

The secreted GUS protein is 602 amino acids long and does not have a canonical leader peptide. A prototypic leader sequence has an amino-terminal positively charged region, a central hydrophobic region, and a more polar carboxy-terminal region (*see*, von Heijne, *J. Membrane Biol. 115*:195-201, 1990) and is generally about 20 amino acids long. However, in both mammalian and bacterial cells, proteins without canonical or identifiable secretory sequences have been found in extracellular or periplasmic spaces.

EXAMPLE 2

PROPERTIES OF SECRETED β-GLUCURONIDASE

Although the screen described above suggests that the *Bacillus* GUS is secreted, the cellular localization of *Bo*GUS is examined. Cellular fractions (*e.g.*, periplasm, spheroplast, supernatant, etc.) are prepared from KW1 cells transformed with pRAJa17.1 or a subfragment that contains the GUS gene and from *E. coli* cells that express β-glucuronidase. GUS activity and β-galactosidase activity is determined for each fraction. The percent of total activity in the periplasm fraction for GUS and β-gal (a non-secreted protein) are calculated; the amount of β-gal activity is considered background and thus is subtracted from the amount of β-glucuronidase activity. In Figure 6, the relative activities of *Bo*GUS and *E. coli* GUS in the periplasm fraction are plotted. As shown, approximately 50% of *Bo*GUS activity is found in the periplasm, whereas less than 10% of *E. coli* GUS activity is present.

The thermal stability of *Bo*GUS and *E. coli* GUS enzymes are determined at 65°C. using an substrate that can be measured by spectrophotometry, for example. One such substrate is p-nitrophenyl β-D-glucuronide (pNPG), which when cleaved by GUS releases the chromophore p-nitrophenol. At a pH greater than its pKa (approximately 7.15), the ionized chromophore absorbs light at 400-420 nm. in the yellow range of visible light. Briefly, reactions are performed in 50 mM NaPO4 pH

7.0, 10 mM 2-ME, 1 mM EDTA, 1 mM pNPG, and 0.1% Triton X-100 at 37°C. The reactions are terminated by the addition of 0.4 ml of 2-amino, 2-methyl propanediol, and absorbance measured at 415 nm against a substrate blank. Under these conditions, the molar extinction coefficient of p-nitorphenol is assumed to be 14,000. One unit is defined as the amount of enzyme that produces 1 nmole of product/min at 37°C.

As shown in Figure 7, *Bo*GUS has a half-life of approximately 16 min, while *E. coli* GUS has a half-life of less than 2 min. Thus, *Bo*GUS is at least 8 times more stable than the *E. coli* GUS. In addition, the catalytic properties of *Bo*GUS are substantially better than the *E. coli* enzyme. The Km is two-fold less and the Vmax is 2.5 times greater.

Table 1

	BoGUS	*E. coli GUS*
Km	70 μM pNPG	150 μM pNPG
Vmax	90 nmoles/min/μg	35 nmoles/min/μg

The turnover number of *Bo*GUS is 2.5 to 5 times higher than *E. coli* GUS at either 37°C or at room temperature (Figures 8 and 9). A turnover number is calculated as nmoles of pNPG converted to p-nitrophenol per min per μg of purified protein.

*Bo*GUS enzyme activity is resistant to inhibition by detergents. Enzyme activity assays are measured in the presence of varying amounts of SDS, Triton X-100, or sarcosyl. As presented in Figure 10, *Bo*GUS was not inhibited or only slightly inhibited (< 20% inhibition) in Triton X-100 and Sarcosyl. In SDS, the enzyme still had substantial activity (60-75% activity). In addition, *Bo*GUS is not inhibited by the end product of the reaction. Activity is determined normally or in the presence of 1 or 10 mM glucuronic acid. No inhibition is seen at either 1 or 10 mM glucuronic acid (Figure 11). The enzyme is also assayed in the presence of organic solvents, dimethylformamide (DMF) and dimethylsulfoxide (DMSO), and high concentrations of NaCl (Figure 12). Only at the highest concentrations of DMF and DMSO (20%) does *Bo*GUS demonstrate inhibition, which is approximately 40% inhibited. In lesser

concentrations of organic solvent and in the presence of 1 M NaCl, *Bo*GUS retains essentially complete activity.

EXAMPLE 3

CONSTRUCTION OF A CODON OPTIMIZED SECRETED β-GLUCURONIDASE

The *Bacillus* GUS gene is codon-optimized for expression in *E. coli*. Codon frequencies for each codon are determined by back translation using ecohigh codons for highly expressed genes of enteric bacteria. These ecohigh codon usages are available from GCG. The most frequently used codon for each amino acid is then chosen for synthesis. In addition, the polyadenylation signal, AATAAA. splice consensus sequences, ATTTA AGGT, and restriction sites that are found in polylinkers are eliminated. Other changes may be made to reduce potential secondary structure. To facilitate cloning in various vectors, four different 5' ends are synthesized: the first, called A0 (shown in Figure 13), uses a sequence comprising an *Nco* I (underlined). *Bgl* II (double underlined), and *Spe* I (italicized) sites (GTCGACCCATGGTAGATCTG*ACTAGT*) are added just 5' to the Leu codon at amino acid 2 in Figure 3. The second one, called AI, adds the native Shine/Dalgarno sequence (GTCGACAGGAGTGCTATC) 5' of the initiator Met codon; the third, called AII, adds a modified Shine/Dalgarno sequence 5' of the initiator Met codon such that a *Nco* I site is added (GTCGACAGGAGTGCTAC); the fourth one, called AIII adds a modified Shine/Dalgarno sequence (GTCGACAGGAGTGCTACCATGGTAGAT) 5' of the Leu codon (residue 2). All of these 5' added sequences contain a *Sal* I site at the extreme 5' end to facilitate construction and cloning. In certain embodiments, to facilitate protein purification, a sequence comprising an *Nhe* I (underlined) site, an *Apa* I (double underlined) site, and encoding hexa-his amino acids at joined at the 3' (COOH-terminus) of the gene.

GCTAGCCATCACCATCACCATCACGTGTGAATTGGTGACCGGGCCC
SerSerHisHisHisHisHisHisVal *

Nucleotide and amino acid sequences of one engineered secretable microbial GUS are shown in Figure 13, and a schematic is shown in Figure 14. The

coding sequence for this protein is assembled in pieces. The sequence is dissected into four fragments, A (bases 1-457); B (bases 458-1012); C (bases 1013-1501); and D (bases 1502-1875). Oligonucleotides (Table 2) that are roughly 80 bases (range 36-100 bases) are synthesized to overlap and create each fragment. The fragments are each cloned separately and the DNA sequence verified. Then, the four fragments are excised and assembled in pLITMUS 39 (New England Biolabs, Beverley, MA), which is a small, high copy number cloning plasmid.

Table 2

Oligo name	Size	Sequence
BoGUS A-1-80T	80	TCGACCCATGGTAGATCTGACTAGTCTGTACCCGATCAACAC CGAGACCCGTGGCGTCTTCGACCTCAATGGCGTCTGGA
BoGUS A-121-200B	80	GGATTTCCTTGGTCACGCCAATGTCATTGTAACTGCTTGGGA CGGCCATACTAATAGTGTCGGTCAGCTTGCTTTCGTAC
BoGUS A-161-240T	80	CCAAGCAGTTACAATGACATTGGCGTGACCAAGGAAATCCGC AACCATATCGGATATGTCTGGTACGAACGTGAGTTCAC
BoGUS A-201-280B	80	GCGGAGCACGATACGCTGATCCTTCAGATAGGCCGGCACCGT GAACTCACGTTCGTACCAGACATATCCGATATGGTTGC
BoGUS A-241-320T	80	GGTGCCGGCCTATCTGAAGGATCAGCGTATCGTGCTCCGCTT CGGCTCTGCAACTCACAAAGCAATTGTCTATGTCAATG
BoGUS A-281-360B	80	AATGGCAGGAATCCGCCCTTGTGCTCCACGACCAGCTCACCA TTGACATAGACAATTGCTTTGTGAGTTGCAGAGCCGAA
BoGUS A-321-400T	80	GTGAGCTGGTCGTGGAGCACAAGGGCGGATTCCTGCCATTCG AAGCGGAAATCAACAACTCGCTGCGTGATGGCATGAAT
BoGUS A-361-460B	100	GTACAGCCCCACCGGTAGGGTGCTATCGTCGAGGATGTTGTC CACGGCGACGGTGACGCGATTCATGCCATCACGCAGCGAGTT GTTGATTTCCGCTTCG
BoGUS A-401-456T	56	CGCGTCACCGTCGCCGTGGACAACATCCTCGACGATAGCACC CTACCGGTGGGGCT
BoGUS A-41-120B	80	CACTTCTCTTCCAGTCCTTTCCCGTAGTCCAGCTTGAAGTTC CAGACGCCATTGAGGTCGAAGACGCCACGGGTCTCGGT
BoGUS A-6-40B	35	TTGATCGGGTACAGACTAGTCAGATCTACCATGGG
BoGUS A-81-160T	80	ACTTCAAGCTGGACTACGGGAAAGGACTGGAAGAGAAGTGGT ACGAAAGCAAGCTGACCGACACTATTAGTATGGCCGTC
BoGUS B-1-80T	80	GTACAGCGAGCGCCACGAAGAGGGCCTCGGAAAAGTCATTCG TAACAAGCCGAACTTCGACTTCTTCAACTATGCAGGCC
BoGUS B-121-200B	80	CTTTGCCTTGAAAGTCCACCGTATAGGTCACAGTCCCGGTTG GGCCATTGAAGTCGGTCACAACCGAGATGTCCTCGACG
BoGUS B-161-240T	80	ACCGGGACTGTGACCTATACGGTGGACTTTCAAGGCAAAGCC GAGACCGTGAAAGTGTCGGTCGTGGATGAGGAAGGCAA
BoGUS B-201-280B	80	CTCCACGTTACCGCTCAGGCCCTCGGTGCTTGCGACCACTTT GCCTTCCTCATCCACGACCGACACTTTCACGGTCTCGG
BoGUS B-241-320T	80	AGTGGTCGCAAGCACCGAGGGCCTGAGCGGTAACGTGGAGAT TCCGAATGTCATCCTCTGGGAACCACTGAACACGTATC

BoGUS B-281-360B	80	GTCAGTCCGTCGTTCACCAGTTCCACTTTGATCTGGTAGAGA TACGTGTTCAGTGGTTCCCAGAGGATGACATTCGGAAT
BoGUS B-321-400T	80	TCTACCAGATCAAAGTGGAACTGGTGAACGACGGACTGACCA TCGATGTCTATGAAGAGCCGTTCGGCGTGCGGACCGTG
BoGUS B-361-440B	80	ACGGTTTGTTGTTGATGAGGAACTTGCCGTCGTTGACTTCCA CGGTCCGCACGCCGAACGGCTCTTCATAGACATCGATG
BoGUS B-401-480T	80	GAAGTCAACGACGGCAAGTTCCTCATCAACAACAAACCGTTC TACTTCAAGGGCTTTGGCAAACATGAGGACACTCCTAT
BoGUS B-41-120B	80	TACGTAAACGGGGTCGTGTAGATTTTCACCGGACGGTGCAGG CCTGCATAGTTGAAGAAGTCGAAGTTCGGCTTGTTACG
BoGUS B-441-520B	80	ATCCATCACATTGCTCGCTTCGTTAAAGCCACGGCCGTTGAT AGGAGTGTCCTCATGTTTGCCAAAGCCCTTGAAGTAGA
BoGUS B-481-555T	75	CAACGGCCGTGGCTTTAACGAAGCGAGCAATGTGATGGATTT CAATATCCTCAAATGGATCGGCGCCAACAGCTT
BoGUS B-5-40B	36	AATGACTTTTCCGAGGCCCTCTTCGTGGCGCTCGCT
BoGUS B-521-559B	39	CCGGAAGCTGTTGGCGCCGATCCATTTGAGGATATTGAA
BoGUS B-81-160T	80	TGCACCGTCCGGTGAAAATCTACACGACCCCGTTTACGTACG TCGAGGACATCTCGGTTGTGACCGACTTCAATGGCCCA
BoGUS C-1-80T	80	CCGGACCGCACACTATCCGTACTCTGAAGAGTTGATGCGTCT TGCGGATCGCGAGGGTCTGGTCGTGATCGACGAGACTC
BoGUS C-121-200B	80	GTTCACGGAGAACGTCTTGATGGTGCTCAAACGTCCGAATCT TCTCCCAGGTACTGACGCGCTCGCTGCCTTCGCCGAGT
BoGUS C-161-240T	80	ATTCGGACGTTTGAGCACCATCAAGACGTTCTCCGTGAACTG GTGTCTCGTGACAAGAACCATCCAAGCGTCGTGATGTG
BoGUS C-201-280B	80	CGCGCCCTCTTCCTCAGTCGCCGCCTCGTTGGCGATGCTCCA CATCACGACGCTTGGATGGTTCTTGTCACGAGACACCA
BoGUS C-241-320T	80	GAGCATCGCCAACGAGGCGGCGACTGAGGAAGAGGGCGCGTA CGAGTACTTCAAGCCGTTGGTGGAGCTGACCAAGGAAC
BoGUS C-281-360B	80	ACAAACAGCACGATCGTGACCGGACGCTTCTGTGGGTCGAGT TCCTTGGTCAGCTCCACCAACGGCTTGAAGTACTCGTA
BoGUS C-321-400T	80	TCGACCCACAGAAGCGTCCGGTCACGATCGTGCTGTTTGTGA TGGCTACCCCGGAGACGGACAAAGTCGCCGAACTGATT
BoGUS C-361-440B	80	CGAAGTACCATCCGTTATAGCGATTGAGCGCGATGACGTCAA TCAGTTCGGCGACTTTGTCCGTCTCCGGGGTAGCCATC
BoGUS C-401-489T	89	GACGTCATCGCGCTCAATCGCTATAACGGATGGTACTTCGAT GGCGGTGATCTCGAAGCGGCCAAAGTCCATCTCCGCCAGGAA TTTCA
BoGUS C-41-120B	80	CCCGTGGTGGCCATGAAGTTGAGGTGCACGCCAACTGCCGGA GTCTCGTCGATCACGACCAGACCCTCGCGATCCGCAAG
BoGUS C-441-493B	53	CGCGTGAAATTCCTGGCGGAGATGGACTTTGGCCGCTTCGAG ATCACCGCCAT
BoGUS C-5-40B	36	ACGCATCAACTCTTCAGAGTACGGATAGTGTGCGGT
BoGUS C-81-160T	80	CGGCAGTTGGCGTGCACCTCAACTTCATGGCCACCACGGGAC TCGGCGAAGGCAGCGAGCGCGTCAGTACCTGGGAGAAG
BoGUS D-1-80T	80	CGCGTGGAACAAGCGTTGCCCAGGAAAGCCGATCATGATCAC TGAGTACGGCGCAGACACCGTTGCGGGCTTTCACGACA
BoGUS D-121-200B	80	TCGCGAAGTCCGCGAAGTTCCACGCTTGCTCACCCACGAAGT TCTCAAACTCATCGAACACGACGTGGTTCGCCTGGTAG

BoGUS D-161-240T	80	TTCGTGGGTGAGCAAGCGTGGAACTTCGCGGACTTCGCGACC TCTCAGGGCGTGATGCGCGTCCAAGGAAACAAGAAGGG
BoGUS D-201-280B	80	GTGCGCGGCGAGCTTCGGCTTGCGGTCACGAGTGAACACGCC CTTCTTGTTTCCTTGGACGCGCATCACGCCCTGAGAGG
BoGUS D-241-320T	80	CGTGTTCACTCGTGACCGCAAGCCGAAGCTCGCCGCGCACGT CTTTCGCGAGCGCTGGACCAACATTCCAGATTTCGGCT
BoGUS D-281-369B	89	CGGTCACCAATTCACACGTGATGGTGATGGTGATGGCTAGCG TTCTTGTAGCCGAAATCTGGAATGTTGGTCCAGCGCTCGCGA AAGAC
BoGUS D-321-373T	53	ACAAGAACGCTAGCCATCACCATCACCATCACGTGTGAATTG GTGACCGGGCC
BoGUS D-41-120B	80	TACTCGACTTGATATTCCTCGGTGAACATCACTGGATCAATG TCGTGAAAGCCCGCAACGGTGTCTGCGCCGTACTCAGT
BoGUS D-5-40B	36	GATCATGATCGGCTTTCCTGGGCAACGCTTGTTCCA
BoGUS D-81-160T	80	TTGATCCAGTGATGTTCACCGAGGAATATCAAGTCGAGTACT ACCAGGCGAACCACGTCGTGTTCGATGAGTTTGAGAAC

The GUS insert from pLITMUS 39 is excised and cloned into the backbone of pLAD-F48, a modular cloning vector derived from pTTQ18 (Amersham). pLAD-F48 (Figure 15) has a lac UV5/ trp hybrid promoter, a Shine-Dalgarno sequence from lac, and a terminator from rrnB.

The AI form of microbial GUS in pLITMUS 39 is transfected into KW1 host *E. coli* cells. Bacterial cells are collected by centrifugation and resuspended in buffer (20 mM $NaPO_4$, pH 7.0, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 0.5 µg/ml leupeptin, 1 µg/ml aprotinin, 0.7 µg/ml pepstatin). This mixture is evenly suspended via a Polytron homogenizer, and the cells are broken open by agitation with glass beads or passage through a microfluidizer. For hexa-His fusion proteins, the lysate is clarified by centrifugation at 50,000 rpm for 45 min and batch absorbed on a Ni-IDA-Sepharose column. The matrix is poured into a column and washed with buffer, typically either 50 mM Tris pH 7.6, 1 mM DTT; 50 mM MES pH 7.0, or IMAC buffer (for hexa-his fusions). The β-glucuronidase protein bound to the matrix is eluted in NaCl-containing buffer.

If GUS is cloned without the HexaHis tail, the lysate is centrifuged at 50,000 rpm for 45 min, and diluted with 20 mM $NaPO_4$, 1 mM EDTA, pH 7.0 (buffer A). The diluted supernatant is then loaded onto a SP-Sepharose or equivalent column, and a linear gradient of 0 to 30% SP Buffer B (1 M NaCl, 20 mM $NaPO_4$, 1 mM EDTA,

pH 7.0) in Buffer A with a total of 6 column volumes is applied. Fractions containing GUS are combined. Further purifications can be performed.

EXAMPLE 4

MUTEINS OF CODON OPTIMIZED β-GLUCURONIDASE

Muteins of the codon-optimized GUS genes are constructed. Each of the four GUS genes described above, A0, AI, AII, and AIII, contain none, one, or four amino acid alterations. The muteins that contain one alteration have a Leu 141 to His codon change. The muteins that contain four alterations have the Leu141 to His change as well as Val138 to Ala, Tyr204 to Asp, and Thr560 to Ala changes. pLITMUS 39 containing these 12 muteins are transfected into KW1. Colonies are tested for secretion of the introduced GUS gene by staining with X-glcA. A white colony indicates undetectable GUS activity, a light blue colony indicates some detectable activity, and a dark blue colony indicates a higher level of detectable activity. As shown in the Table below, when GUS has the four mutations, no GUS activity is detectable. When GUS has a single Leu 141 to His mutation, three of the four constructs exhibit no GUS activity, while the AI construct exhibits a low level of GUS activity. All constructs exhibit GUS activity when no mutations are present. Thus, the Leu 141 to His mutation dramatically affects the activity of GUS.

Number of Mutations	GUS construct			
	A0	AI	AII	AIII
4	white	white	white	white
1	white	light blue	white	white
0	light blue	dark blue	light blue	light blue

EXAMPLE 5

EXPRESSION OF MICROBIAL β-GLUCURONIDASES

IN YEAST, PLANTS AND *E. COLI*

A series of expression vector constructs of three different GUS genes, *Ec*GUS, *Bacillus* GUS, and the A0 version of codon-optimized *Bacillus* GUS, are prepared and tested for enzymatic activity in *E. coli*, yeast, and plants (rice, Millin variety, and *Arabidopsis*). The GUS genes are cloned in vectors that either contain a signal peptide suitable for the host or do not contain a signal peptide. The *E. coli* vector contains a sequence encoding a *pel*B signal peptide, the yeast vectors contain a sequence encoding either an invertase or Mat alpha signal peptide, and the plant vectors contain a sequence encoding either a glycine-rich protein (GRP) or extensin signal peptide.

Invertase signal sequence:
ATGCTTTTGC AAGCCTTCCT TTTCCTTTTG GCTGGTTTTG CAGCCAAAAT ATCTGCAATG

Mat alpha signal sequence:
ATGAGATTTC CTTCAATTTT TACTGCAGTT TTATTCGCAG CATCCTCCGC ATTAGCTGCT
CCAGTCAACA CTACAACAGA AGATGAAACG GCACAAATTC CGGCTGAAGC TGTCATCGGT
TACTTAGATT TAGAAGGGGA TTTCGATGTT GCTGTTTTGC CATTTTCCAA CAGCACAAAT
AACGGGTTAT TGTTTATAAA TACTACTATT GCCAGCATTG CTGCTAAAGA AGAAGGGGTA
TCTTTGGATA AAAGAGAG

Extensin signal sequence
CATGGGAAAA ATGGCTTCTC TATTTGCCAC ATTTTTAGTG GTTTTAGTGT CACTTAGCTT
AGCTTCTGAA AGCTCAGCAA ATTATCAA

GRP signal sequence
CATGGCTACT ACTAAGCATT TGGCTCTTGC CATCCTTGTC CTCCTTAGCA TTGGTATGAC
CACCAGTGCA AGAACCCTCC TA

The GUS genes are cloned into each of these vectors using standard recombinant techniques of isolation of a GUS-gene containing fragment and ligation into an appropriately restricted vector. The recombinant vectors are then transfected into the appropriate host and transfectants are tested for GUS activity.

As shown in the Table below, all tested transfectants exhibited GUS activity (indicated by a +). Moreover, similar results are obtained regardless of the presence or absence of a signal peptide.

GUS	*E. coli*		Yeast			Plants		
	No SP*	pelB	No SP	Invertase	Mat α	No SP	GRP	Extensin
EcGUS	+	NT	+	+	–	+	NT	NT

AI GUS	+	NT	+	+	+	NT	NT	NT
Bacillus GUS	+	NT	+	+	+	+	+	+

*; SP=signal peptide; NT=not tested

EXAMPLE 6

EXPRESSION OF LOW-CYSTEINE *E. COLI* β-GLUCURONIDASE

The *E. coli* GUS protein has nine cysteine residues, whereas. human GUS has four and *Bacillus* GUS has one. Low-cysteine muteins of *E. coli* GUS are constructed to provide a form of *Ec*GUS that is secretable.

Single and multiple Cys muteins are constructed by site-directed mutagenesis techniques. Eight of the nine cysteine residues in EcGUS are changed to the corresponding residue found in human GUS based on alignment of the two protein sequences. One of the EcGUS cysteine residues, amino acid 463, aligns with a cysteine residue in human GUS and was not altered. The corresponding amino acids between EcGUS and human GUS are shown below.

Identifier	EcGUS Cys residue no.	Human GUS corresponding amino acid
A	28	Asn
B	133	Ala
C	197	Ser
D	253	Glu
E	262	Ser
F	442	Phe
G	448	Tyr
H	463	Cys
I	527	Lys

The mutein GUS genes are cloned into a pBS backbone. The mutations are confirmed by diagnostic restriction site changes and by DNA sequence analysis. Recombinant vectors are transfected into KW1 and GUS activity assayed by staining with X-glcA (5-bromo, 4-chloro, 3-indolyl-β-D-glucuronide).

As shown in the Table below, when the Cys residues at 443 (F). 449 (G), and 528 (I) are altered, GUS activity is greatly or completely diminished. In contrast, when the N-terminal five Cys residues (A, B, C, D, and E) are altered, GUS activity remains detectable.

Cys changes	GUS activity
A	yes
B	yes
C	yes
I	no
D, E	yes
F, G	no
C, D, E	yes
B, C, D, E	yes
A, B, C, D, E	yes
A, B, C, D, E, I	no

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

We claim:

1. An isolated nucleic acid molecule comprising a nucleic acid sequence encoding a secreted form of microbial β-glucuronidase.

2. The nucleic acid molecule of claim 1, wherein the microbe is *Bacillus*.

3. The nucleic acid molecule of claim 1, wherein the nucleic acid sequence comprises nucleotides 1662-3467 of Figure 1 or hybridizes under stringent conditions to the complement of the sequence comprising nucleotides 1662-3467 of Figure 1, and which encodes a functional β-glucuronidase.

4. The nucleic acid molecule of claim 1, wherein the nucleic acid molecule encodes the amino acid sequence of Figure 3, or a variant thereof, and which encodes a functional β-glucuronidase.

5. The nucleic acid molecule of claim 4, further comprising a second nucleic acid molecule that encodes the amino acid sequence of Figure 4A, or a variant thereof, wherein the second nucleic acid molecule is fused to the 5' end of the molecule of claim 4.

6. The nucleic acid molecule of claim 1, wherein the nucleic acid molecule encodes a low-cysteine variant of *E. coli* β-glucuronidase.

7. The nucleic acid molecule of claim 6, wherein at least one of the cysteine residues at 28, 133, 197, 253, and 262 are changed.

8. An isolated secreted form of microbial β-glucuronidase.

9. The β-glucuronidase of claim 8, wherein the microbe is *Bacillus*.

10. The β-glucuronidase of claim 8, wherein the β-glucuronidase comprises a low-cysteine variant of *E. coli* β-glucuronidase.

11. The β-glucuronidase of claim 10, wherein the β-glucuronidase has at least one alteration of the cysteine residue at 28, 133, 197, 253, and 262.

12. The β-glucuronidase of claim 9, wherein the β-glucuronidase is encoded by a nucleic acid molecule comprising nucleotides 1662-3467 of Figure 1 or by a nucleic acid molecule that hybridizes under stringent conditions to the complement of nucleotides 1662-3467 of Figure 1 and which encodes a functional β-glucuronidase.

13. The β-glucuronidase of claim 9, comprising the amino acid sequence of Figure 3, or a variant thereof, and which encodes a functional β-glucuronidase.

14. An isolated nucleic acid molecule encoding a membrane-bound form of microbial β-glucuronidase.

15. The nucleic acid molecule of claim 14, wherein the sequence encoding β-glucuronidase is fused to a sequence encoding a membrane-spanning peptide.

16. An isolated nucleic acid molecule encoding a cytoplasmic form of microbial β -glucuronidase.

17. The nucleic acid molecule of claim 16, wherein the sequence encoding β-glucuronidase is fused to a sequence encoding a cytoplasm-directing peptide.

18. The nucleic acid molecule of claim 17, wherein the cytoplasm-directing peptide is Lys-Asp-Glu-Leu.

19. A vector, comprising a nucleic acid molecule encoding a microbial β-glucuronidase, wherein the β-glucuronidase sequence is in operative linkage with a promoter element.

20. The vector of claim 19, wherein the promoter element is a promoter selected from the group consisting of a developmental type-specific promoter, a tissue type-specific promoter, a cell type-specific promoter and an inducible promoter.

21. The vector of claim 20, wherein the promoter element is selected from a group consisting of a promoter functional in a plant cell, a promoter functional in a bacterium, a promoter functional in an animal cell and a promoter functional in a fungal cell.

22. The vector of claim 19, wherein the vector is functional in a bacterium.

23. The vector of claim 19, wherein the vector is a binary *Agrobacterium tumefaciens* plasmid vector.

24. The vector of claim 19, further comprising a nucleic acid sequence encoding a product of a gene of interest or portion thereof.

25. The vector of claim 24, wherein the product is a protein.

26. A host cell containing the vector according to claim 19.

27. The host cell of claim 26, wherein the host cell is selected from the group consisting of a plant cell, an insect cell, a fungal cell, an animal cell and a bacterial cell.

28. A method of producing a secreted form of microbial β-glucuronidase, comprising:

(a) introducing a vector encoding a secreted form of microbial β-glucuronidase in operative linkage with a promoter; and

(b) growing the host cell under conditions wherein the microbial β-glucuronidase is expressed.

29. The method according to claim 28, further comprising isolating the β-glucuronidase from cell supernatant or periplasm.

30. The method according to claim 28, wherein the host cell is a bacterial cell.

31. A method of introducing a controller element into a host cell, comprising introducing into the host cell a vector construct, the vector construct comprising nucleic acid sequence encoding a secreted form of microbial β-glucuronidase and a nucleic acid sequence of the controller element,

wherein the nucleic acid sequence encoding the β-glucuronidase (a) encodes a protein comprising the amino acid sequence of Figure 3 or (b) hybridizes under stringent conditions to the complement of nucleotides 1662-3467 of Figure 1, and which encodes a functional beta-glucuronidase, and wherein the nucleic acid sequence encoding β-glucuronidase is in operative linkage with the controller element.

32. The method according to claim 31, wherein the host cell is selected from the group consisting of a plant cell, an animal cell, an insect cell, a fungal cell and a bacterial cell.

33. The method according to claim 31, wherein the vector construct is a binary *Agrobacterium* vector.

34. The method according to claim 31, wherein the controller element is selected from the group consisting of a promoter, an enhancer, an operator, a ribosome binding site, a signal peptide sequence, a chloroplast targeting sequence, a mitochondrial localization sequence, a nucleus targeting sequence and an intron.

35. The method according to claim 34, wherein the controller element is functional in a plant cell.

36. The method according to claim 34, wherein the controller element is a promoter selected from the group consisting of a developmental type-specific promoter, a tissue type-specific promoter, a cell type-specific promoter and an inducible promoter.

37. A method of monitoring expression of a gene of interest or a portion thereof in a host cell, comprising:

(a) introducing into the host cell a vector construct, the vector construct comprising nucleic acid sequence encoding a secreted form of microbial β-glucuronidase and nucleic acid sequence encoding a product of the gene of interest or a portion thereof:

wherein the nucleic acid sequence encoding the microbial β-glucuronidase (a) encodes a protein comprising the amino acid sequence of Figure 3 or (b) hybridizes under stringent conditions to the complement of nucleotides 1662-3467 of Figure 1 and which encodes a functional β-glucuronidase, and

(b) detecting the presence of secreted microbial β-glucuronidase. thereby monitoring expression of the gene of interest.

38. The method according to claim 37, wherein the host cell is selected from the group consisting of a plant cell, an animal cell, an insect cell, a fungal cell and a bacterial cell.

39. The method according to claim 37, wherein the product is a protein.

40. The method according to claim 37, wherein the vector construct further comprises a promoter.

41. The method according to claim 37, wherein the nucleic acid sequence encoding the product and the nucleic acid sequence encoding β-glucuronidase are in operative linkage with the same promoter.

42. A method of monitoring activity of a controller element in a host cell, comprising:

(a) introducing into the host cell a vector construct, the vector construct comprising nucleic acid sequence encoding a secreted form of microbial β-glucuronidase and a nucleic acid sequence of the controller element;

wherein the nucleic acid sequence encoding the β-glucuronidase (a) encodes a protein comprising the amino acid sequence of Figure 3 or (b) hybridizes under stringent conditions to the complement of nucleotides 1662-3467 of Figure 1 and which encodes a functional β-glucuronidase, and

wherein the nucleic acid sequence encoding β-glucuronidase is in operative linkage with the controller element; and

(b) detecting the presence of secreted β-glucuronidase, thereby monitoring activity of the controller element.

43. The method according to claim 42, wherein the host cell is selected from the group consisting of a plant cell, an animal cell, an insect cell, a fungal cell and a bacterial cell.

44. The method according to claim 42, wherein the vector construct is a binary *Agrobacterium* vector.

45. The method according to claim 42, wherein the controller element is

selected from the group consisting of a promoter, an enhancer, an operator, a ribosome binding site, a signal peptide sequence, a chloroplast targeting sequence, a mitochondrial localization sequence, a nucleus targeting sequence and an intron.

46. The method according to claim 42, wherein the controller element is a promoter functional in a plant cell.

47. A method for transforming a host cell with a gene of interest or portion thereof, comprising:

(a) introducing into the host cell a vector construct, the vector construct comprising nucleic acid sequence encoding a secreted form of microbial β-glucuronidase and nucleic acid sequence encoding a product of the gene of interest or a portion thereof, such that the vector construct integrates into the genome of the host cell;

wherein the nucleic acid sequence encoding β-glucuronidase (i) encodes a protein comprising the amino acid sequence of Figure 3 or (ii) hybridizes under stringent conditions to the complement of nucleotides 1662-3467 of Figure 1 and which encodes a functional β-glucuronidase; and

(b) detecting the presence of secreted β-glucuronidase, thereby establishing that the host cell is transformed.

48. The method according to claim 47, wherein the host cell is selected from the group consisting of a plant cell, an animal cell, an insect cell, a fungal cell and a bacterial cell.

49. The method according to claim 47, wherein the vector construct is a binary *Agrobacterium* vector.

50. The method according to claim 47, wherein the product is a protein.

51. The method according to claim 47, wherein the vector construct further

comprises a promoter.

52. The method according to claim 47, wherein the gene of interest and β-glucuronidase are under control of the same promoter.

53. A method of positive selection for a transformed cell, comprising:

(a) introducing into a host cell a vector construct, the vector construct comprising nucleic acid sequence encoding a microbial β-glucuronidase;

wherein the nucleic acid sequence encoding β-glucuronidase (a) encodes a protein comprising the amino acid sequence of Figure 3 or (b) hybridizes under stringent conditions to the complement of nucleotides 1662-3467 of Figure 1 and which encodes a functional β-glucuronidase; and

(b) exposing the host cell to the sample comprising a glucuronide, wherein the glucuronide is cleaved by the β-glucuronidase, such that the compound is released, wherein the compound is required for cell growth.

54. The method of claim 53, further comprising introducing into the host cell a vector construct comprising a nucleic acid sequence encoding a microbial glucuronide permease.

55. The method according to claim 53, wherein the host cell is selected from the group consisting of a plant cell, an animal cell, an insect cell, a fungal cell and a bacterial cell.

56. A method of producing a transgenic plant that expresses a secreted form of microbial β-glucuronidase, comprising:

(a) introducing a vector according to claim 19 into an embryogenic plant cell; and

(b) producing a plant from the embryogenic plant cell, wherein the plant expresses the β-glucuronidase.

57. The method of claim 56, wherein the step of introducing is by *Agrobacterium* co-cultivation or bombardment.

58. A transgenic plant cell comprising the vector according to claim 19.

59. A transgenic plant comprising the plant cell of claim 58.

60. A transgenic insect cell comprising the vector according to claim 19.

61. A transgenic insect comprising the insect cell of claim 60.

62. A method for identifying an organism that secretes β-glucuronidase, comprising:

(a) culturing the organism or cells from the organism in a medium containing a substrate for β-glucuronidase, wherein the cleaved substrate is detectable; and

(b) detecting the cleaved substrate in the medium;

therefrom identifying an organism that secretes β-glucuronidase.

63. The method of claim 62, wherein the organism is a bacterium isolated from soil, skin, or fecal matter.

64. The method of claim 62, wherein the organism is *Bacillus*.

65. A method for releasing a compound from a glucuronide in a sample, comprising exposing the sample to a microbial β-glucuronidase, wherein the glucuronide is cleaved by the β-glucuronidase, therefrom releasing the compound, provided that the microbial β-glucuronidase is not wild-type *E. coli* β-glucuronidase.

66. The method of claim 65, further comprising detecting the compound.

67. The method of claim 65, wherein the β-glucuronidase comprises the amino acid sequence presented in Figure 3.

68. The method of claim 65, wherein the sample is a biological fluid selected from the group consisting of blood, saliva, urine, apocrine secretion, synovial fluid and amniotic fluid.

69. The method of claim 65, wherein the compound is a toxin, a hormone, or a drug.

70. A method of releasing a compound from a glucuronide exposed to a host cell, comprising:

(a) introducing into the host cell a vector construct, the vector construct comprising a nucleic acid molecule encoding a microbial β-glucuronidase; and

(b) exposing the host cell to the glucuronide, wherein the glucuronide is cleaved by the beta-glucuronidase, such that the compound is released.

71. The method of claim 70, wherein the nucleic acid molecule encoding beta-glucuronidase (a) encodes a protein comprising the amino acid sequence as depicted in Figure 3 or (b) hybridizes under stringent conditions to the complement of nucleotides 1662-3467 as depicted in Figure 1 and which encodes a functional beta-glucuronidase.

72. The method of claim 70, further comprising introducing into the host cell a vector construct comprising a nucleic acid molecule encoding a glucuronide permease.

73. The method according to claim 70, wherein the compound is an auxin.

74. The method according to claim 73, wherein the auxin is indole-3-ethanol.

75. The method according to claim 70, wherein the compound is a hormone or a toxin.

76. The method according to claim 70, wherein the compound is required for cell growth.

77. The method according to claim 70, wherein the host cell is a plant cell, an animal cell, a fungal cell, or a bacterial cell.

78. The method according to claim 70, wherein the compound is a herbicide.

79. A method of detecting binding between two or more molecules, comprising:

(a) contacting the two or more molecules in a reaction mixture for a time sufficient to allow binding, wherein one of the molecules is conjugated to a hapten;

(b) contacting the bound molecules from step (a) with a microbial β-glucuronidase, wherein the β-glucuronidase binds to the hapten; and

(c) detecting the β-glucuronidase.

80. The method of claim 79, wherein the β-glucuronidase is fused to a protein that binds the hapten.

81. The method of claim 80, wherein the protein is streptavidin and the hapten is biotin.

82. A method of detecting binding between two or more molecules, comprising:

(a) contacting the two or more molecules in a reaction mixture for a time sufficient to allow binding, wherein one of the

molecules is conjugated to microbial β-glucuronidase;

(b) detecting the microbial β-glucuronidase;

therefrom detecting binding between the two molecules.

83. A kit, comprising a microbial β-glucuronidase, provided that the microbial β-glucuronidase is not *E. coli* glucuronidase.

MICROBIAL β-GLUCURONIDASE GENES, GENE PRODUCTS AND USES THEREOF

ABSTRACT OF THE DISCLOSURE

Genes encoding microbial β-glucuronidase and protein that is secreted and its uses are provided.

FIG. 1A

```
   1 aagcttgagc ggtcatatct gccccaccca cgctcgcgtc ccaatttatt catgacttgc
  61 tgggtaggcg ggaaaaactt ttcggccgct gcttcagtac tctccgcaat gaaaccatgg
 121 gaatgggaag caaccggcaa ctttgacacg tcatgacctg catgagcggc tgccttttta
 181 tagagcctca caagtggctc aaactgcagt gggcggcccc caataatggc tagaactagt
 241 ggcaagccaa gcaggccagc acggatgacg gaatcctgac tgccgccact gccaatccaa
 301 acaggtaaag gatcctgaac aggtcttggg tacacaccga gattctggat ggccggccga
 361 tgtccgcctt tccagttcac cttctcggac tcccgtattt ttaacaaaag ctccagtttc
 421 tcatcgaata attcatcata gtcttttaaa tcatagccaa acagcggaaa ggattcgata
 481 aaggagcctc gccctgccat aatctctgca cgtccattcg atatggcatc gagggtagca
 541 aaatcctgaa atactcggac tggatcagca gaagatagaa ccgtcaccgc acttgttaaa
 601 cgaatccgtt ttgtctgcca agcagcggca gccaatagaa ctgctggaga tgatgccgca
 661 aaatcttcgc gatgatgctc accaacacca aagacatcca gcaatacctc gtctgcgagt
 721 acaatttcct caaccacttc ccgaatccgt tgggaatgac tcatcacttc accggtttca
 781 acatccggtg ttgtctctac gaacgtgctt atacctattt ccacaatcat tacctcctat
 841 gtataatcgt ttgctcttgt gccaaagcta tatgaatttc ttattattgc tgacttttтc
 901 accatatata taaatgaaag aatatttcaa acgttattat cttatatttt cctatttatt
 961 tcaaaaaaat tgtttaacta gcgaaagtag gactaccata caaaatgccc atgttgaaca
1021 aaacaaagca ttttttccgc cgttgtttca tacataagaa aggtgcatga ttaagaaatt
1081 ctataaaggc gcaccgagga ggacaatgat gattcaacaa accgttatga ttaacagaga
1141 agcaggttta tatgctcagc cagtcaatca attagtgcaa acagcttcac aattcaatgc
1201 tgatatcttt ctttcataca aaggacgaaa ggttagtgtg aaatcggtac tcggcgtttt
1261 atcgttagcg atacctaaac aggccgaaat tatcttagaa gtttccggag atgatgaaaa
1321 agaagcactc aaaggggtta tcaatgcgtt ggagaaatta gactagggtt ttccctttt
1381 aatagggaat caccttgaca ttgaaaaagt ataagaaaat gaaaatagga aaaaccaatg
1441 acttaagggg agtctctatt ggaaagagac tccccttatt caacattaga acgaaattag
1501 agcctttact tttctttcaa cttttcatcc cgatactttt ttgtaatagt ttttttcatt
1561 aataatacaa gtcctgattt tgcaagaata atccttttta gataaaaata tctatgctaa
1621 taataacatg taaccactta catttaaaaa ggagtgctat catgttatat ccaatcaata
1681 cagaaacccg aggagttttt gatttaaatg gggtctggaa ttttaaatta gattacggca
1741 aaggactgga agaaaagtgg tatgaatcaa aactgacaga taccatatca atggctgtac
1801 cttcctccta taatgatatc ggtgttacga aggaaattcg aaaccatatc ggctatgtat
1861 ggtacgagcg tgaatttacc gttcctgctt atttaaaaga tcagcgcatc gtcctgcgtt
1921 ttggttcagc aacacataag gctattgtat acgttaacgg agaactagta gttgaacaca
1981 aaggcggctt cttaccgttt gaggcagaaa taaacaacag cttaagagac ggaatgaatc
2041 gtgtaacagt agcggttgat aatattttag atgattctac gctcccagtt gggctatata
2101 gtgaaagaca tgaagaaggt ttgggaaaag tgattcgtaa taaacctaat tttgacttct
2161 ttaactatgc aggcttacat cgtcctgtaa aaatttatac aacccctttt acctatgttg
2221 aggatatatc ggttgtaacc gattttaacg gtccaacggg aacagttacg tatacagttg
2281 attttcaggg taaggcagaa accgtaaagg ttagtgtagt tgatgaagaa gggaaagttg
2341 ttgcttcaac tgaaggcctc tctggtaatg ttgagattcc taacgttatc ctttgggaac
2401 ctttaaatac ctatctctat caaattaaag ttgagttagt aaatgatggt ctaactattg
2461 atgtatacga agagccattt ggagttcgaa ccgttgaagt aaacgacggg aaattcctca
2521 ttaataacaa accattttat tttaaagggt tcggaaaaca cgaggatact ccaataaatg
2581 gaagaggctt taatgaagca tcaaatgtaa tggattttaa tattttgaaa tggatcggtg
2641 cgaattcctt tcggacggcg cactatcctt attctgaaga actgatgcgg ctcgcagatc
2701 gtgaagggtt agtcgtcata gatgaaaccc cagcagttgg tgttcatttg aactttatgg
2761 caacgactgg tttgggcgaa ggttcagaga gagtgagtac ttgggaaaaa atccggacct
2821 ttgaacatca tcaagatgta ctgagagagc tggtttctcg tgataaaaac caccctctg
2881 ttgtcatgtg gtcgattgca aatgaagcgg ctacggaaga agaaggcgct tatgaatact
2941 ttaagccatt agttgaatta acgaaagaat tagatccaca aaaacgccca gttaccattg
```

FIG. 1B

3001 ttttgttcgt aatggcgaca ccagaaacag ataaagtggc ggagttaatt gatgtgattg
3061 cattgaatcg atacaacggc tggtattttg atgggggtga tcttgaagcc gcgaaagtcc
3121 accttcgtca ggaatttcat gcgtggaata aacgctgtcc aggaaaacct ataatgataa
3181 cagagtatgg ggctgatacc gtagctggtt ttcatgatat tgatccggtt atgtttacag
3241 aagagtatca ggttgaatat taccaagcaa atcatgtagt atttgatgaa tttgagaact
3301 ttgttggcga gcaggcctgg aattttgcag actttgctac aagccagggt gtcatgcgtg
3361 ttcaaggtaa caaaaaaggt gttttcacac gcgaccgcaa accaaaatta gcagcacatg
3421 ttttccgcga acgttggaca aacatcccgg atttcggtta taaaaattaa taaaaagctg
3481 gttctccaat aggaggccag ctttttaca tggatacaat ggttgtaaat taaaaaccct
3541 cttcattttt tatataaaaa tgaagagggt tttaattttt taaatgttat tacatttttt
3601 ctaagcccac tcatacaata tgggactttg gatagcatgg gaaacagctt ttttagactg
3661 tagtttcca gtcagctgca aatttttcaa ttccttggtc tgttaaagga tgttttgata
3721 attgctcaat taccttgaat ggaatcgttg caatatgagc tccagccatc gccacacgtg
3781 taacatgatc tggatgacga acagatgcag caatgatttg tgaatccaag ttttgaatct
3841 ggaacatctt agcaattttt gcgactaatt ctacaccatc ttcgttaata tcatctaacc
3901 tgcctaagaa tggtgaaaca taagttgcac ctgctcgtgc tgccagcaat gcctggttaa
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4081 taatgagctc atttgcttca gcaatcattt gatcagctgt caaagcatta ggtgttactt
4141 cggcagaaac agactcaacc tcgggtacgg cattaaggat ttcagcaata cggtcctcaa
4201 atttcacgcc ctctttagct actaaagaag ggttcgttgt tactcctgat aacacgccaa
4261 ttttataggc ttttttgatt tcctctaggt tggcagtatc gataaaaaat ttcataatgt
4321 ttttcctcca atttttagta aagtaatttt tcgtttctaa agcatgtccc caacggaaat
4381 taggttattg aatataatat aggttacttt ccgttaccat aatataacta tccgacaata
4441 atcgtcaagt aaaatgtctt gaattaaaga tatttatttt tttcaaaaga tactatttac
4501 tttactttat tgataagaat tcacgcatcc taactaggat ggcgtgaatt aactttcctt
4561 attcgacaac tccatctcgt tattgtgagg gagtacttcc tgtttctttt ttaaatactc
4621 ttgcaaagta ggagggatca tcatagccaa tcgtccaggc gatttcctct acggataaat
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4801 gtgctgccaa ttcttcttta ttgatttgct tattataaaa acttagcagg tgttcaatca
4861 ccctttgggt catgtttgta tagctactta atgaattgga aatgattaaa tcgcaatatt
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4981 cgtaagcata tttttccgaa attcgatgaa taatgatggc aggtacttgg ctgtttcttg
5041 ctgacgtacg gagaagtatt taatataatc gctacatttt ttagtctgcg caacggctga
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5221 tgactagttc cgcttcttca tccacgttaa gatgattcac tttattgtga ataggacggt
5281 tgtttttatc agaaacaatg acaaacgggg taatctcttc ctccaacatg tgtggaaact
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5461 atagattcgg tgtcttatca aaatatggtc cgatgataat ggtgtaggct gcctgctttt
5521 gggtgaagga atatccgaaa tagtgtaagt cccattcgtt tatataagaa tataattggt
5581 cctgatgctt catttttcg aacaaattca gtggatcttc tttctctgaa cctggcataa
5641 atagcgggat tgcaatgatt tcatgatggt acacaaactc cccattttga tctaaaacat
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6001 cgtcctcccg caggacattg agtaagctt

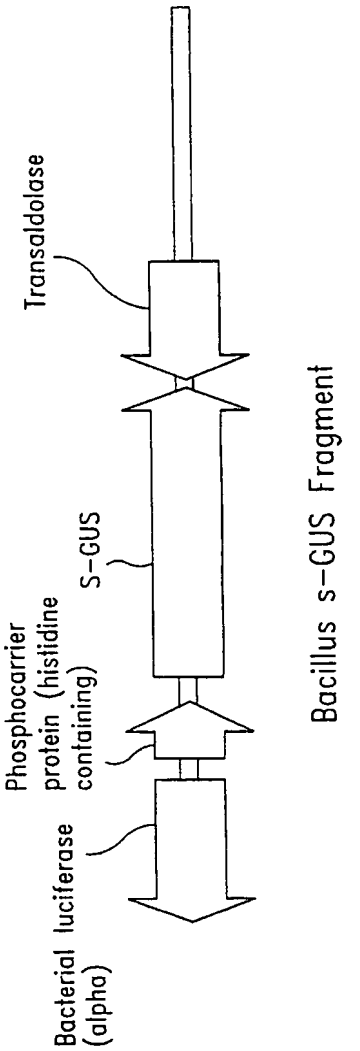
Transaldolase
S-GUS
Phosphocarrier protein (histidine containing)
Bacterial luciferase (alpha)
Bacillus s-GUS Fragment

Fig. 2

FIG. 3

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  1  MLYPINTETR GVFDLNGVWN FKLDYGKGLE EKWYESKLTD TISMAVPSSY
 51  NDIGVTKEIR NHIGYVWYER EFTVPAYLKD QRIVLRFGSA THKAIVYVNG
101  ELVVEHKGGF LPFEAEINNS LRDGMNRVTV AVDNILDDST LPVGLYSERH
151  EEGLGKVIRN KPNFDFFNYA GLHRPVKIYT TPFTYVEDIS VVTDFNGPTG
201  TVTYTVDFQG KAETVKVSVV DEEGKVVAST EGLSGNVEIP NVILWEPLNT
251  YLYQIKVELV NDGLTIDVYE EPFGVRTVEV NDGKFLINNK PFYFKGFGKH
301  EDTPINGRGF NEASNVMDFN ILKWIGANSF RTAHYPYSEE LMRLADREGL
351  VVIDETPAVG VHLNFMATTG LGEGSERVST WEKIRTFEHH QDVLRELVSR
401  DKNHPSVVMW SIANEAATEE EGAYEYFKPL VELTKELDPQ KRPVTIVLFV
451  MATPETDKVA ELIDVIALNR YNGWYFDGGD LEAAKVHLRQ EFHAWNKRCP
501  GKPIMITEYG ADTVAGFHDI DPVMFTEEYQ VEYYQANHVV FDEFENFVGE
551  QAWNFADFAT SQGVMRVQGN KKGVFTRDRK PKLAAHVFRE RWTNIPDFGY
601  KN
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FIG. 4A

```
    MetLeuIleIleThrCysAsnHisLeuHisLeuLysArgSerAlaIle
    ATGCTAATAATAACATGTAACCACTTACATTTAAAAAGGAGTGCTATC

    MetLeuTyrProIleAsnThrGluThrArgGlyValPheAspLeuAsnGl
1   ATGTTATATCCAATCAATACAGAAACCCGAGGAGTTTTTGATTTAAATGG

    yValTrpAsnPheLysLeuAspTyrGlyLysGlyLeuGluGluLysTrpT
51  GGTCTGGAATTTTAAATTAGATTACGGCAAAGGACTGGAAGAAAAGTGGT

    yrGluSerLysLeuThrAspThrIleSerMetAlaValProSerSerTyr
101 ATGAATCAAAACTGACAGATACCATATCAATGGCTGTACCTTCCTCCTAT

    AsnAspIleGlyValThrLysGluIleArgAsnHisIleGlyTyrValTr
151 AATGATATCGGTGTTACGAAGGAAATTCGAAACCATATCGGCTATGTATG

    pTyrGluArgGluPheThrValProAlaTyrLeuLysAspGlnArgIleV
201 GTACGAGCGTGAATTTACCGTTCCTGCTTATTTAAAAGATCAGCGCATCG

    alLeuArgPheGlySerAlaThrHisLysAlaIleValTyrValAsnGly
251 TCCTGCGTTTTGGTTCAGCAACACATAAGGCTATTGTATACGTTAACGGA

    GluLeuValValGluHisLysGlyGlyPheLeuProPheGluAlaGluIl
301 GAACTAGTAGTTGAACACAAAGGCGGCTTCTTACCGTTTGAGGCAGAAAT

    eAsnAsnSerLeuArgAspGlyMetAsnArgValThrValAlaValAspA
351 AAACAACAGCTTAAGAGACGGAATGAATCGTGTAACAGTAGCGGTTGATA

    snIleLeuAspAspSerThrLeuProValGlyLeuTyrSerGluArgHis
401 ATATTTTAGATGATTCTACGCTCCCAGTTGGGCTATATAGTGAAAGACAT

    GluGluGlyLeuGlyLysValIleArgAsnLysProAsnPheAspPhePh
451 GAAGAAGGTTTGGGAAAAGTGATTCGTAATAAACCTAATTTTGACTTCTT

    eAsnTyrAlaGlyLeuHisArgProValLysIleTyrThrThrProPheT
501 TAACTATGCAGGCTTACATCGTCCTGTAAAAATTTATACAACCCCTTTTA

    hrTyrValGluAspIleSerValValThrAspPheAsnGlyProThrGly
551 CCTATGTTGAGGATATATCGGTTGTAACCGATTTTAACGGTCCAACGGGA

    ThrValThrTyrThrValAspPheGlnGlyLysAlaGluThrValLysVa
601 ACAGTTACGTATACAGTTGATTTTCAGGGTAAGGCAGAAACCGTAAAGGT

    lSerValValAspGluGluGlyLysValValAlaSerThrGluGlyLeuS
651 TAGTGTAGTTGATGAAGAAGGGAAAGTTGTTGCTTCAACTGAAGGCCTCT
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FIG. 4B

```
      erGlyAsnValGluIleProAsnValIleLeuTrpGluProLeuAsnThr
701   CTGGTAATGTTGAGATTCCTAACGTTATCCTTTGGGAACCTTTAAATACC

      TyrLeuTyrGlnIleLysValGluLeuValAsnAspGlyLeuThrIleAs
751   TATCTCTATCAAATTAAAGTTGAGTTAGTAAATGATGGTCTAACTATTGA

      pValTyrGluGluProPheGlyValArgThrValGluValAsnAspGlyL
801   TGTATACGAAGAGCCATTTGGAGTTCGAACCGTTGAAGTAAACGACGGGA

      ysPheLeuIleAsnAsnLysProPheTyrPheLysGlyPheGlyLysHis
851   AATTCCTCATTAATAACAAACCATTTTATTTTAAAGGGTTCGGAAAACAC

      GluAspThrProIleAsnGlyArgGlyPheAsnGluAlaSerAsnValMe
901   GAGGATACTCCAATAAATGGAAGAGGCTTTAATGAAGCATCAAATGTAAT

      tAspPheAsnIleLeuLysTrpIleGlyAlaAsnSerPheArgThrAlaH
951   GGATTTTAATATTTTGAAATGGATCGGTGCGAATTCCTTTCGGACGGCGC

      isTyrProTyrSerGluGluLeuMetArgLeuAlaAspArgGluGlyLeu
1001  ACTATCCTTATTCTGAAGAACTGATGCGGCTCGCAGATCGTGAAGGGTTA

      ValValIleAspGluThrProAlaValGlyValHisLeuAsnPheMetAl
1051  GTCGTCATAGATGAAACCCCAGCAGTTGGTGTTCATTTGAACTTTATGGC

      aThrThrGlyLeuGlyGluGlySerGluArgValSerThrTrpGluLysI
1101  AACGACTGGTTTGGGCGAAGGTTCAGAGAGAGTGAGTACTTGGGAAAAAA

      leArgThrPheGluHisHisGlnAspValLeuArgGluLeuValSerArg
1151  TCCGGACCTTTGAACATCATCAAGATGTACTGAGAGAGCTGGTTTCTCGT

      AspLysAsnHisProSerValValMetTrpSerIleAlaAsnGluAlaAl
1201  GATAAAAACCACCCCTCTGTTGTCATGTGGTCGATTGCAAATGAAGCGGC

      aThrGluGluGluGlyAlaTyrGluTyrPheLysProLeuValGluLeuT
1251  TACGGAAGAAGAAGGCGCTTATGAATACTTTAAGCCATTAGTTGAATTAA

      hrLysGluLeuAspProGlnLysArgProValThrIleValLeuPheVal
1301  CGAAAGAATTAGATCCACAAAAACGCCCAGTTACCATTGTTTTGTTCGTA

      MetAlaThrProGluThrAspLysValAlaGluLeuIleAspValIleAl
1351  ATGGCGACACCAGAAACAGATAAAGTGGCGGAGTTAATTGATGTGATTGC

      aLeuAsnArgTyrAsnGlyTrpTyrPheAspGlyGlyAspLeuGluAlaA
1401  ATTGAATCGATACAACGGCTGGTATTTTGATGGGGGTGATCTTGAAGCCG
```

FIG. 4C

```
      laLysValHisLeuArgGlnGluPheHisAlaTrpAsnLysArgCysPro
1451  CGAAAGTCCACCTTCGTCAGGAATTTCATGCGTGGAATAAACGCTGTCCA

      GlyLysProIleMetIleThrGluTyrGlyAlaAspThrValAlaGlyPh
1501  GGAAAACCTATAATGATAACAGAGTATGGGGCTGATACCGTAGCTGGTTT

      eHisAspIleAspProValMetPheThrGluGluTyrGlnValGluTyrT
1551  TCATGATATTGATCCGGTTATGTTTACAGAAGAGTATCAGGTTGAATATT

      yrGlnAlaAsnHisValValPheAspGluPheGluAsnPheValGlyGlu
1601  ACCAAGCAAATCATGTAGTATTTGATGAATTTGAGAACTTTGTTGGCGAG

      GlnAlaTrpAsnPheAlaAspPheAlaThrSerGlnGlyValMetArgVa
1651  CAGGCCTGGAATTTTGCAGACTTTGCTACAAGCCAGGGTGTCATGCGTGT

      lGlnGlyAsnLysLysGlyValPheThrArgAspArgLysProLysLeuA
1701  TCAAGGTAACAAAAAAGGTGTTTTCACACGCGACCGCAAACCAAAATTAG

      laAlaHisValPheArgGluArgTrpThrAsnIleProAspPheGlyTyr
1751  CAGCACATGTTTTCCGCGAACGTTGGACAAACATCCCGGATTTCGGTTAT

      LysAsn
1801  AAAAAT
```

FIG. 5

```
BGUS      ------MLYPINTETRGVFDLNGVWNFKLDYG----KGLEEKWYESKLTDT---ISMAVP 47
HGUS      LGLQGGMLYPQESPSRECKELDGLWSFRADFSDNRRRGFEEQWYRRPLWESGPTVDMPVP 60
EGUS      ------MLRPVETPTREIKKLDGLWAFSLDREN---CGIDQRWWESALQESR---AIAVP 48

BGUS      SSYNDIGVTKEIRNHIGYVWYEREFTVPAYLKD---QRIVLRFGSATHKAIVYVNGELVV 104
HGUS      SSFNDISQDWRLRHFVGWVWYEREVILPERWTQDLRTRVVLRIGSAHSYAIVWVNGVDTL 120
EGUS      GSFNDQFADADIRNYAGNVWYQREVFIPKGWAG---QRIVLRFDAVTHYGKVWVNNQEVM 105

BGUS      EHKGGFLPFEAEINNSLRDG----MNRVTVAVDNILDDSTLPVG-LYSERHEEGLGKVIR 159
HGUS      EHEGGYLPFEADISNLVQVGPLPSRLRITIAINNTLTPTTLPPGTIQYLTDTSKYPKGYF 180
EGUS      EHQGGYTPFEADVTPYVIAG---KSVRITVCVNNELNWQTIPPG--MVITDENGKKK--- 157

BGUS      -NKPNFDFFNYAGLHRPVKIYTTPFTYVEDISVVTDFNGPT--GTVTYTVDFQG-KAETV 215
HGUS      VQNTYFDFFNYAGLQRSVLLYTTPTTYIDDITVTTSVEQDS--GLVNYQISVKGSNLFKL 238
EGUS      -QSYFHDFFNYAGIHRSVMLYTTPNTWVDDITVVTHVAQDCNHASVDWQVVANG----DV 212

BGUS      KVSVVDEEGKVVASTEGLSGNVEIPNVILWEP-----LNTYLYQIKVELVNDGLT---ID 267
HGUS      EVRLLDAENKVVANGTGTQGQLKVPGVSLWWPYLMHERPAYLYSLEVQLTAQTSLGPVSD 298
EGUS      SVELRDADQQVVATGQGTSGTLQVVNPHLWQP-----GEGYLYELCVTAKSQTEC----D 263

BGUS      VYEEPFGVRTVEVNDGKFLINNKPFYFKGFGKHEDTPINGRGFNEASNVMDFNILKWIGA 327
HGUS      FYTLPVGIRTVAVTKSQFLINGKPFYFHGVNKHEDADIRGKGFDWPLLVKDFNLLRWLGA 358
EGUS      IYPLRVGIRSVAVKGEQFLINHKPFYFTGFGRHEDADLRGKGFDNVLMVHDHALMDWIGA 323

BGUS      NSFRTAHYPYSEELMRLADREGLVVIDETPAVGVHLNFMATTGLGEGSERVSTWEKIR-- 385
HGUS      NAFRTSHYPYAEEVMQMCDRYGIVVIDECPGVGLAL----------P------QFFNNV 401
EGUS      NSYRTSHYPYAEEMLDWADEHGIVVIDETAAVGFNLSLGIGFEAGNKPKELYSEEAVNGE 383

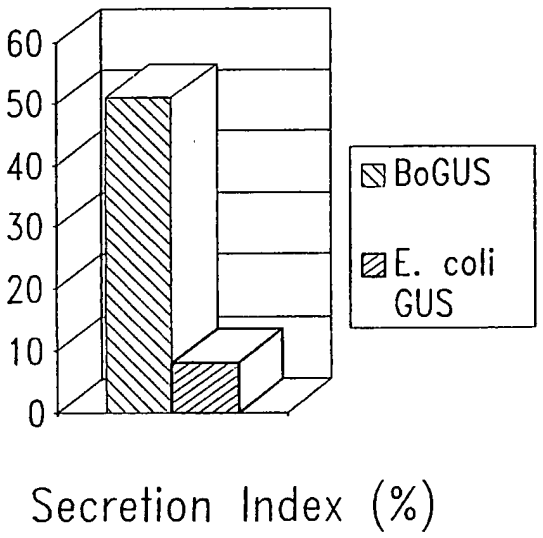
BGUS      TFEHHQDVLRELVSRDKNHPSVVMWSIANEAATEEEGAYEYFKPLVELTKELDPQKRPVT 445
HGUS      SLHHHMQVMEEVVRRDKNHPAVVMWSVANEPASHLESAGYYLKMVIAHTKSLDPS-RPVT 460
EGUS      TQQAHLQAIKELIARDKNHPSVVMWSIANEPDTRPQGAREYFAPLAEATRKLDPT-RPIT 442

BGUS      IVLFVMATPETDKVAELIDVIALNRYNGWYFDGGDLEAAKVHLRQEFHAWNKRCPGKPIM 505
HGUS      FVS--NSNYAADKGAPYVDVICLNSYYSWYHDYGHLELIQLQLATQFENWYKKYQ-KPII 517
EGUS      CVNVMFCDAHTDTISDLFDVLCLNRYYGWYVQSGDLETAEKVLEKELLAWQEKLH-QPII 501

BGUS      ITEYGADTVAGFHDIDPVMFTEEYQVEYYQANHVVFD--EFENFVGEQAWNFADFATSQG 563
HGUS      QSEYGAETIAGFHQDPPLMFTEEYQKSLLEQYHLGLDQKRRKYVVGELIWNFADFMTEQS 577
EGUS      ITEYGVDTLAGLHSMYTDMWSEEYQCAWLDMYHRVFD--RVSAVVGEQVWNFADFATSQG 559

BGUS      VMRVQGNKKGVFTRDRKPKLAAHVFRERWTNIPDFGYKN----- 602
HGUS      PTRVLGNKKGIFTRQRQPKSAAFLLRERYWKIAN-ET------- 613
EGUS      ILRVGGNKKGIFTRDRKPKSAAFLLQKRWTGMNFGEKPQQGGKQ 603
```

*Bo*GUS is secreted in *E. coli*



Cellular fractions were assayed for glucuronidase and galactosidase activity

Secretion Index was calculated as follows:

- percent of total activity in the periplasm fraction for glucuronidase and galactosidase was calculated
- galactosidase value was subtracted from glucuronidase as "contamination"

Fig. 6

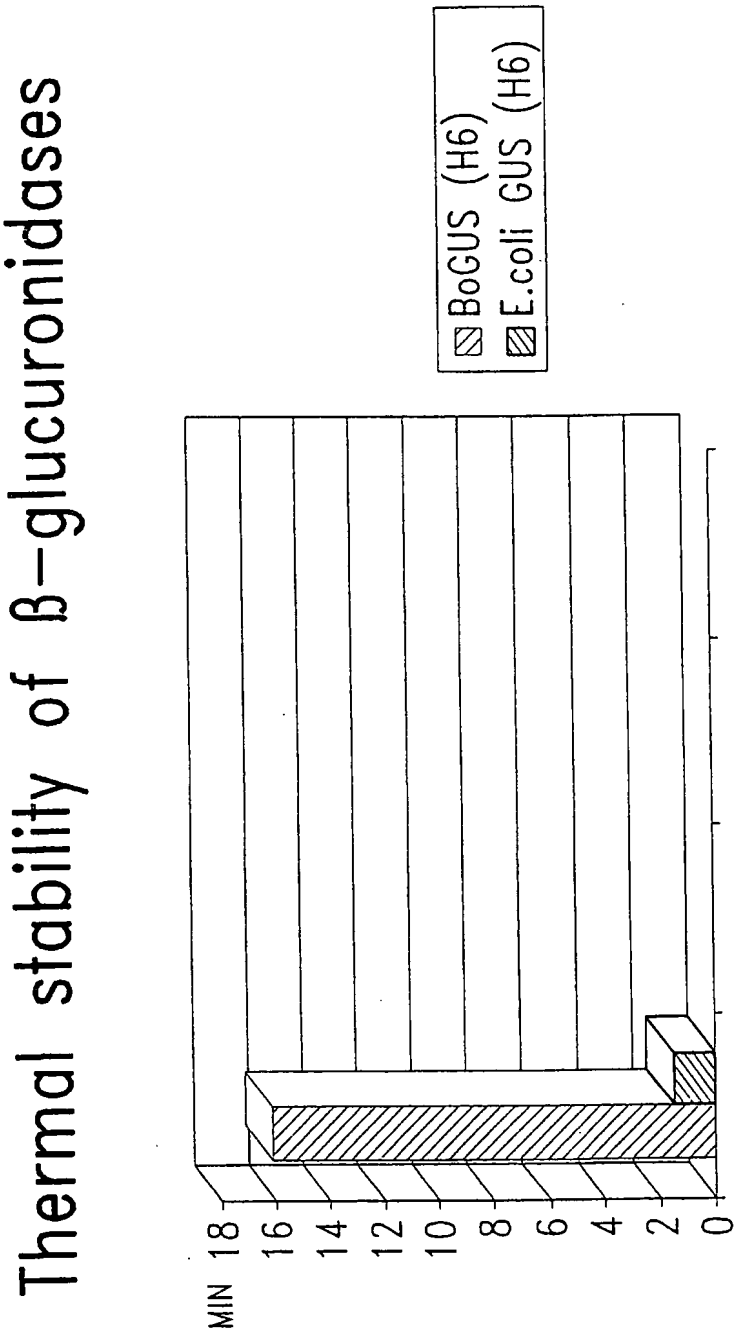

Thermal stability of β-glucuronidases
BoGUS (H6)
E.coli GUS (H6)
MIN 18
16
14
12
10
8
6
4
2
0
Half-life at 65°C

Fig. 7

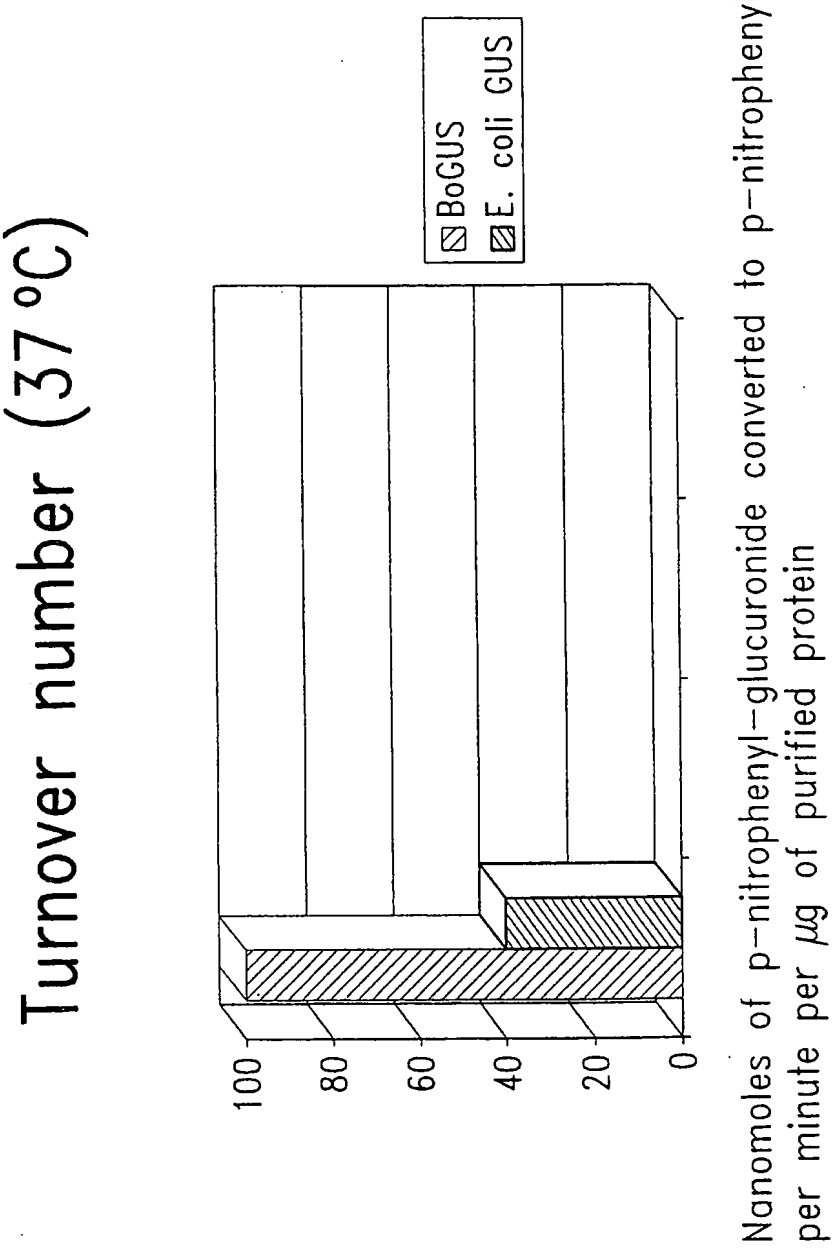

Turnover number (37 °C)
BoGUS
E. coli GUS
100
80
60
40
20
0
Nanomoles of p-nitrophenyl-glucuronide converted to p-nitrophenyl per minute per μg of purified protein

Fig. 8

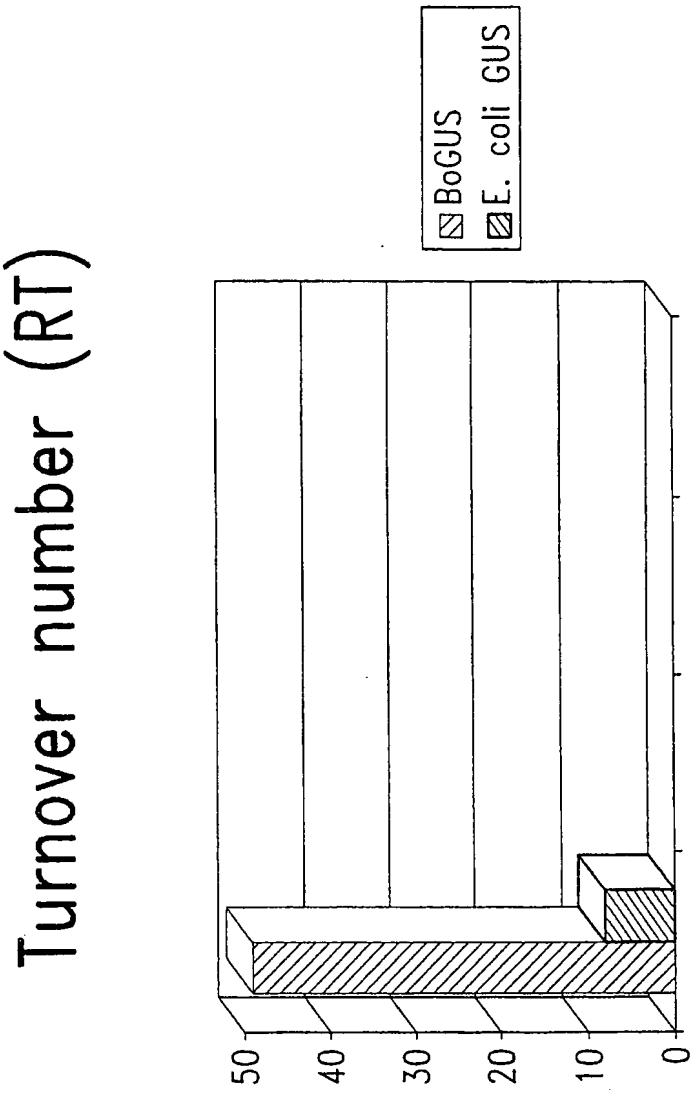

Turnover number (RT)
BoGUS
E. coli GUS
50
40
30
20
10
0
Nanomoles of p-nitrophenyl-glucuronide converted to p-nitrophenyl per minute per μg of purified protein

Fig. 9

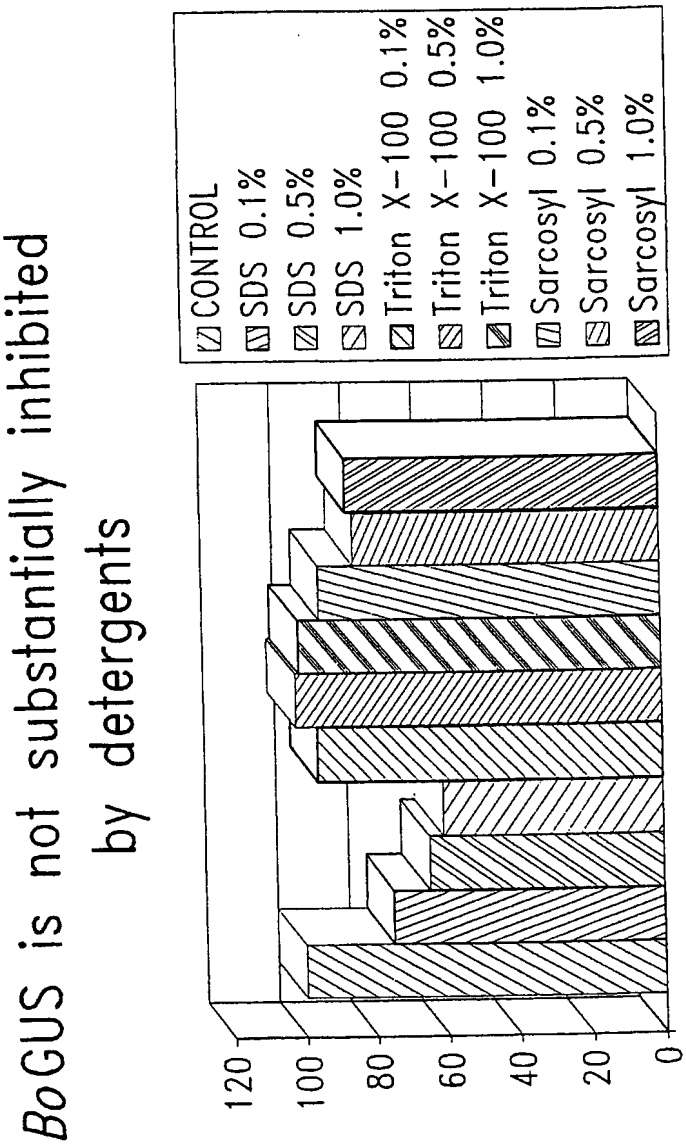

BoGUS is not substantially inhibited by detergents
CONTROL
SDS 0.1%
SDS 0.5%
SDS 1.0%
Triton X-100 0.1%
Triton X-100 0.5%
Triton X-100 1.0%
Sarcosyl 0.1%
Sarcosyl 0.5%
Sarcosyl 1.0%
120
100
80
60
40
20
0

Fig. 10

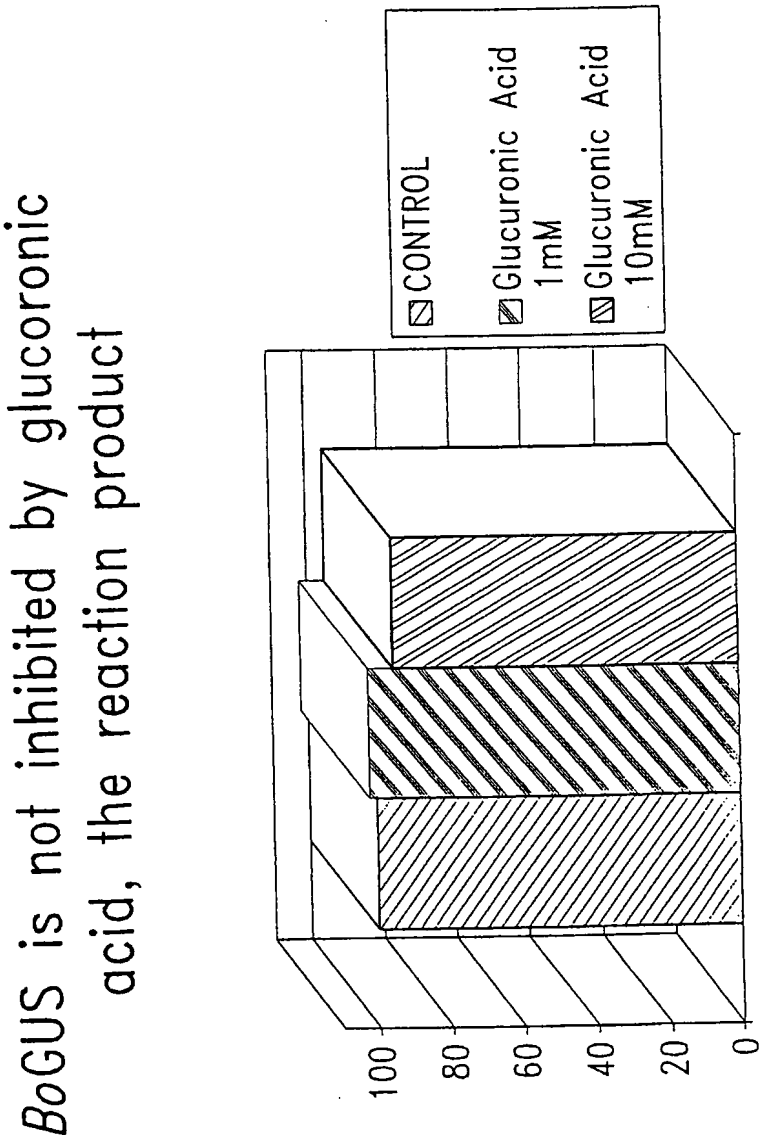

BoGUS is not inhibited by glucoronic acid, the reaction product
CONTROL
Glucuronic Acid 1mM
Glucuronic Acid 10mM
100
80
60
40
20
0

Fig. 11

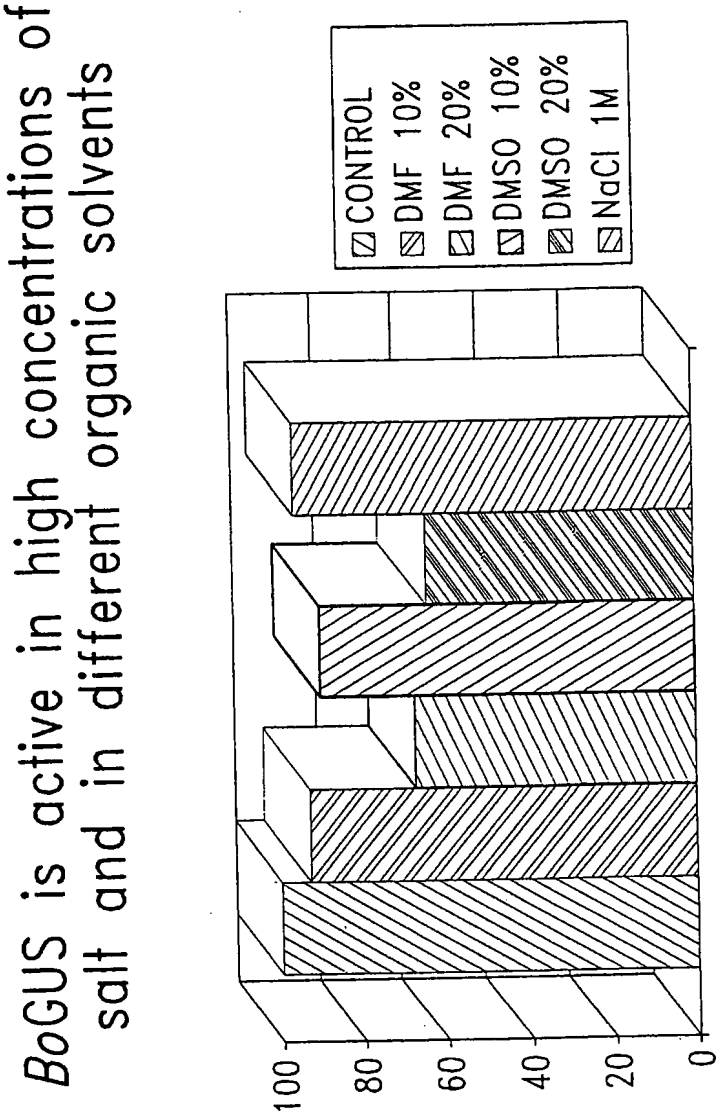

BoGUS is active in high concentrations of salt and in different organic solvents
CONTROL
DMF 10%
DMF 20%
DMSO 10%
DMSO 20%
NaCl 1M
100
80
60
40
20
0

Fig. 12

FIG. 13A

```
  +1                                   M  V   D  L   T   S  L  Y
                              SalI   NcoI    BglII
2451  ATACGACTCA CTAGTGGGTC GACCCATGGT AGATCTGACT AGTCTGTACC
      TATGCTGAGT GATCACCCAG CTGGGTACCA TCTAGACTGA TCAGACATGG

  +1  P  I  N  T   E  T  R   G  V  F  D   L  N  G   V  W  N
2501  CGATCAACAC CGAGACCCGT GGCGTCTTCG ACCTCAATGG CGTCTGGAAC
      GCTAGTTGTG GCTCTGGGCA CCGCAGAAGC TGGAGTTACC GCAGACCTTG

  +1   F  K  L  D   Y  G  K   G  L  E   E  K  W  Y   E  S  K
2551  TTCAAGCTGG ACTACGGGAA AGGACTGGAA GAGAAGTGGT ACGAAAGCAA
      AAGTTCGACC TGATGCCCTT TCCTGACCTT CTCTTCACCA TGCTTTCGTT

  +1   L  T  D   T  I  S  M   A  V  P   S  S  Y   N  D  I
2601  GCTGACCGAC ACTATTAGTA TGGCCGTCCC AAGCAGTTAC AATGACATTG
      CGACTGGCTG TGATAATCAT ACCGGCAGGG TTCGTCAATG TTACTGTAAC

  +1  G  V  T  K   E  I  R   N  H  I  G   Y  V  W   Y  E  R
2651  GCGTGACCAA GGAAATCCGC AACCATATCG GATATGTCTG GTACGAACGT
      CGCACTGGTT CCTTTAGGCG TTGGTATAGC CTATACAGAC CATGCTTGCA

  +1   E  F  T  V   P  A  Y   L  K  D   Q  R  I  V   L  R  F
2701  GAGTTCACGG TGCCGGCCTA TCTGAAGGAT CAGCGTATCG TGCTCCGCTT
      CTCAAGTGCC ACGGCCGGAT AGACTTCCTA GTCGCATAGC ACGAGGCGAA

  +1    G  S  A   T  H  K  A   I  V  Y   V  N  G   E  L  V
2751  CGGCTCTGCA ACTCACAAAG CAATTGTCTA TGTCAATGGT GAGCTGGTCG
      GCCGAGACGT TGAGTGTTTC GTTAACAGAT ACAGTTACCA CTCGACCAGC

  +1  V  E  H  K   G  G  F   L  P  F  E   A  E  I   N  N  S
2801  TGGAGCACAA GGGCGGATTC CTGCCATTCG AAGCGGAAAT CAACAACTCG
      ACCTCGTGTT CCCGCCTAAG GACGGTAAGC TTCGCCTTTA GTTGTTGAGC

  +1   L  R  D  G   M  N  R   V  T  V   A  V  D  N   I  L  D
2851  CTGCGTGATG GCATGAATCG CGTCACCGTC GCCGTGGACA ACATCCTCGA
      GACGCACTAC CGTACTTAGC GCAGTGGCAG CGGCACCTGT TGTAGGAGCT

  +1    D  S  T   L  P  V  G   L  Y  S   E  R  H   E  E  G
2901  CGATAGCACC CTCCCGGTGG GGCTGTACAG CGAGCGCCAC GAAGAGGGCC
      GCTATCGTGG GAGGGCCACC CCGACATGTC GCTCGCGGTG CTTCTCCCGG

  +1  L  G  K  V   I  R  N   K  P  N  F   D  F  F   N  Y  A
2951  TCGGAAAAGT CATTCGTAAC AAGCCGAACT TCGACTTCTT CAACTATGCA
      AGCCTTTTCA GTAAGCATTG TTCGGCTTGA AGCTGAAGAA GTTGATACGT

  +1   G  L  H  R   P  V  K   I  Y  T   T  P  F  T   Y  V  E
3001  GGCCTGCACC GTCCGGTGAA AATCTACACG ACCCCGTTTA CGTACGTCGA
      CCGGACGTGG CAGGCCACTT TTAGATGTGC TGGGGCAAAT GCATGCAGCT
```

FIG. 13B

```
   +1   D  I  S  V  V  T  D  F  N  G  P  T  G  T  V  T
3051  GGACATCTCG GTTGTGACCG ACTTCAATGG CCCAACCGGG ACTGTGACCT
      CCTGTAGAGC CAACACTGGC TGAAGTTACC GGGTTGGCCC TGACACTGGA

   +1 Y  T  V  D  F  Q  G  K  A  E  T  V  K  V  S  V  V
3101  ATACGGTGGA CTTTCAAGGC AAAGCCGAGA CCGTGAAAGT GTCGGTCGTG
      TATGCCACCT GAAAGTTCCG TTTCGGCTCT GGCACTTTCA CAGCCAGCAC

   +1  D  E  E  G  K  V  V  A  S  T  E  G  L  S  G  N  V
3151  GATGAGGAAG GCAAAGTGGT CGCAAGCACC GAGGGCCTGA GCGGTAACGT
      CTACTCCTTC CGTTTCACCA GCGTTCGTGG CTCCCGGACT CGCCATTGCA

   +1   E  I  P  N  V  I  L  W  E  P  L  N  T  Y  L  Y
3201  GGAGATTCCG AATGTCATCC TCTGGGAACC ACTGAACACG TATCTCTACC
      CCTCTAAGGC TTACAGTAGG AGACCCTTGG TGACTTGTGC ATAGAGATGG

   +1 Q  I  K  V  E  L  V  N  D  G  L  T  I  D  V  Y  E
3251  AGATCAAAGT GGAACTGGTG AACGACGGAC TGACCATCGA TGTCTATGAA
      TCTAGTTTCA CCTTGACCAC TTGCTGCCTG ACTGGTAGCT ACAGATACTT

   +1  E  P  F  G  V  R  T  V  E  V  N  D  G  K  F  L  I
3301  GAGCCGTTCG GCGTGCGGAC CGTGGAAGTC AACGACGGCA AGTTCCTCAT
      CTCGGCAAGC CGCACGCCTG GCACCTTCAG TTGCTGCCGT TCAAGGAGTA

   +1   N  N  K  P  F  Y  F  K  G  F  G  K  H  E  D  T
3351  CAACAACAAA CCGTTCTACT TCAAGGGCTT TGGCAAACAT GAGGACACTC
      GTTGTTGTTT GGCAAGATGA AGTTCCCGAA ACCGTTTGTA CTCCTGTGAG

   +1 P  I  N  G  R  G  F  N  E  A  S  N  V  M  D  F  N
3401  CTATCAACGG CCGTGGCTTT AACGAAGCGA GCAATGTGAT GGATTTCAAT
      GATAGTTGCC GGCACCGAAA TTGCTTCGCT CGTTACACTA CCTAAAGTTA

   +1  I  L  K  W  I  G  A  N  S  F  R  T  A  H  Y  P  Y
3451  ATCCTCAAAT GGATCGGCGC CAACAGCTTC CGGACCGCAC ACTATCCGTA
      TAGGAGTTTA CCTAGCCGCG GTTGTCGAAG GCCTGGCGTG TGATAGGCAT

   +1   S  E  E  L  M  R  L  A  D  R  E  G  L  V  V  I
3501  CTCTGAAGAG TTGATGCGTC TTGCGGATCG CGAGGGTCTG GTCGTGATCG
      GAGACTTCTC AACTACGCAG AACGCCTAGC GCTCCCAGAC CAGCACTAGC

   +1 D  E  T  P  A  V  G  V  H  L  N  F  M  A  T  T  G
3551  ACGAGACTCC GGCAGTTGGC GTGCACCTCA ACTTCATGGC CACCACGGGA
      TGCTCTGAGG CCGTCAACCG CACGTGGAGT TGAAGTACCG GTGGTGCCCT

   +1  L  G  E  G  S  E  R  V  S  T  W  E  K  I  R  T  F
3601  CTCGGCGAAG GCAGCGAGCG CGTCAGTACC TGGGAGAAGA TTCGGACGTT
      GAGCCGCTTC CGTCGCTCGC GCAGTCATGG ACCCTCTTCT AAGCCTGCAA

   +1   E  H  H  Q  D  V  L  R  E  L  V  S  R  D  K  N
3651  TGAGCACCAT CAAGACGTTC TCCGTGAACT GGTGTCTCGT GACAAGAACC
      ACTCGTGGTA GTTCTGCAAG AGGCACTTGA CCACAGAGCA CTGTTCTTGG
```

FIG. 13C

```
  +1 H  P  S  V  V  M  W  S  I  A  N  E  A  A  T  E  E
3701 ATCCAAGCGT CGTGATGTGG AGCATCGCCA ACGAGGCGGC GACTGAGGAA
     TAGGTTCGCA GCACTACACC TCGTAGCGGT TGCTCCGCCG CTGACTCCTT

  +1 E  G  A  Y  E  Y  F  K  P  L  V  E  L  T  K  E  L
3751 GAGGGCGCGT ACGAGTACTT CAAGCCGTTG GTGGAGCTGA CCAAGGAACT
     CTCCCGCGCA TGCTCATGAA GTTCGGCAAC CACCTCGACT GGTTCCTTGA

  +1  D  P  Q  K  R  P  V  T  I  V  L  F  V  M  A  T
3801 CGACCCACAG AAGCGTCCGG TCACGATCGT GCTGTTTGTG ATGGCTACCC
     GCTGGGTGTC TTCGCAGGCC AGTGCTAGCA CGACAAACAC TACCGATGGG

  +1 P  E  T  D  K  V  A  E  L  I  D  V  I  A  L  N  R
3851 CGGAGACGGA CAAAGTCGCC GAACTGATTG ACGTCATCGC GCTCAATCGC
     GCCTCTGCCT GTTTCAGCGG CTTGACTAAC TGCAGTAGCG CGAGTTAGCG

  +1 Y  N  G  W  Y  F  D  G  G  D  L  E  A  A  K  V  H
3901 TATAACGGAT GGTACTTCGA TGGCGGTGAT CTCGAAGCGG CCAAAGTCCA
     ATATTGCCTA CCATGAAGCT ACCGCCACTA GAGCTTCGCC GGTTTCAGGT

  +1  L  R  Q  E  F  H  A  W  N  K  R  C  P  G  K  P
3951 TCTCCGCCAG GAATTTCACG CGTGGAACAA GCGTTGCCCA GGAAAGCCGA
     AGAGGCGGTC CTTAAAGTGC GCACCTTGTT CGCAACGGGT CCTTTCGGCT

  +1 I  M  I  T  E  Y  G  A  D  T  V  A  G  F  H  D  I
4001 TCATGATCAC TGAGTACGGC GCAGACACCG TTGCGGGCTT TCACGACATT
     AGTACTAGTG ACTCATGCCG CGTCTGTGGC AACGCCCGAA AGTGCTGTAA

  +1 D  P  V  M  F  T  E  E  Y  Q  V  E  Y  Y  Q  A  N
4051 GATCCAGTGA TGTTCACCGA GGAATATCAA GTCGAGTACT ACCAGGCGAA
     CTAGGTCACT ACAAGTGGCT CCTTATAGTT CAGCTCATGA TGGTCCGCTT

  +1  H  V  V  F  D  E  F  E  N  F  V  G  E  Q  A  W
4101 CCACGTCGTG TTCGATGAGT TTGAGAACTT CGTGGGTGAG CAAGCGTGGA
     GGTGCAGCAC AAGCTACTCA AACTCTTGAA GCACCCACTC GTTCGCACCT

  +1 N  F  A  D  F  A  T  S  Q  G  V  M  R  V  Q  G  N
4151 ACTTCGCGGA CTTCGCGACC TCTCAGGGCG TGATGCGCGT CCAAGGAAAC
     TGAAGCGCCT GAAGCGCTGG AGAGTCCCGC ACTACGCGCA GGTTCCTTTG

  +1 K  K  G  V  F  T  R  D  R  K  P  K  L  A  A  H  V
4201 AAGAAGGGCG TGTTCACTCG TGACCGCAAG CCGAAGCTCG CCGCGCACGT
     TTCTTCCCGC ACAAGTGAGC ACTGGCGTTC GGCTTCGAGC GGCGCGTGCA

  +1  F  R  E  R  W  T  N  I  P  D  F  G  Y  K  N
                                                 NheI
                                                 ~~~~
4251 CTTTCGCGAG CGCTGGACCA ACATTCCAGA TTTCGGCTAC AAGAACGCTA
     GAAAGCGCTC GCGACCTGGT TGTAAGGTCT AAAGCCGATG TTCTTGCGAT
```

FIG. 13D

```
          NheI              PmlI        BstEII
          ~~                ~~~~~~      ~~~~~~~~
4301  GCCATCACCA TCACCATCAC GTGTGAATTG GTGACCG
      CGGTAGTGGT AGTGGTAGTG CACACTTAAC CACTGGC
```

>BGUS sequence (from pTANE95m)

MLYPINTETRGVFDLNGVWNFKLDYGKGLEEKWYESKLTDTISMAVPSSYNDIGVTKEIRNHIGYVWYEREFT
VPAYLKDQRIVLRFGSATHKAIVYVNGELVVEHKGGFLPFEAEINNSLRDGMNRVTVAVDNILDDSTLPVGLY
SERHEEGLGKVIRNKPNFDFFNYAGLHRPVKIYTTPFTYVEDISVVTDFNGPTGTVTYTVDFQGKAETVKVSV
VDEEGKVVASTEGLSGNVEIPNVILWEPLNTYLYQIKVELVNDGLTIDVYEEPFGVRTVEVNDGKFLINNKPF
YFKGFGKHEDTPINGRGFNEASNVMDFNILKWIGANSFRTAHYPYSEELMRLADREGLVVIDETPAVGVHLNF
MATTGLGEGSERVSTWEKIRTFEHHQDVLRELVSRDKNHPSVVMWSIANEAATEEEGAYEYFKPLVELTKELD
PQKRPVTIVLFVMATPETDKVAELIDVIALNRYNGWYFDGGDLEAAKVHLRQEFHAWNKRCPGKPIMITEYGA
DTVAGFHDIDPVMFTEEYQVEYYQANHVVFDEFENFVGEQAWNFADFATSQGVMRVQGNKKGVFTRDRKPKLA
AHVFRERWTNIPDFGYKN

>EGUS sequence (from GUSACORRECTED)

MLRPVETPTREIKKLDGLWAFSLDRENCGIDQRWWESALQESRAIAVPGSFNDQFADADIRNYAGNVWYQREV
FIPKGWAGQRIVLRFDAVTHYGKVWVNNQEVMEHQGGYTPFEADVTPYVIAGKSVRITVCVNNELNWQTIPPG
MVITDENGKKKQSYFHDFFNYAGIHRSVMLYTTPNTWVDDITVVTHVAQDCNHASVDWQVVANGDVSVELRDA
DQQVVATGQGTSGTLQVVNPHLWQPGEGYLYELCVTAKSQTECDIYPLRVGIRSVAVKGEQFLINHKPFYFTG
FGRHEDADLRGKGFDNVLMVHDHALMDWIGANSYRTSHYPYAEEMLDWADEHGIVVIDETAAVGFNLSLGIGF
EAGNKPKELYSEEAVNGETQQAHLQAIKELIARDKNHPSVVMWSIANEPDTRPQGAREYFAPLAEATRKLDPT
RPITCVNVMFCDAHTDTISDLFDVLCLNRYYGWYVQSGDLETAEKVLEKELLAWQEKLHQPIIITEYGVDTLA
GLHSMYTDMWSEEYQCAWLDMYHRVFDRVSAVVGEQVWNFADFATSQGILRVGGNKKGIFTRDRKPKSAAFLL
QKRWTGMNFGEKPQQGGKQ

>HGUS (from SWISS-PROT: P08236)

LGLQGGMLYPQESPSRECKELDGLWSFRADFSDNRRRGFEEQWYRRPLWESGPTVDMPVPSSFNDISQDWRLR
HFVGWVWYEREVILPERWTQDLRTRVVLRIGSAHSYAIVWVNGVDTLEHEGGYLPFEADISNLVQVGPLPSRL
RITIAINNTLTPTTLPPGTIQYLTDTSKYPKGYFVQNTYFDFFNYAGLQRSVLLYTTPTTYIDDITVTTSVEQ
DSGLVNYQISVKGSNLFKLEVRLLDAENKVVANGTGTQGQLKVPGVSLWWPYLMHERPAYLYSLEVQLTAQTS
LGPVSDFYTLPVGIRTVAVTKSQFLINGKPFYFHGVNKHEDADIRGKGFDWPLLVKDFNLLRWLGANAFRTSH
YPYAEEVMQMCDRYGIVVIDECPGVGLALPQFFNNVSLHHHMQVMEEVVRRDKNHPAVVMWSVANEPASHLES
AGYYLKMVIAHTKSLDPSRPVTFVSNSNYAADKGAPYVDVICLNSYYSWYHDYGHLELIQLQLATQFENWYKK
YQKPIIQSEYGAETIAGFHQDPPLMFTEEYQKSLLEQYHLGLDQKRRKYVVGELIWNFADFMTEQSPTRVLGN
KKGIFTRQRQPKSAAFLLRERYWKIANET

CLUSTAL W (1.74) Multiple Sequence Alignments

Sequence 1: HGUS 613 aa
Sequence 2: EGUS 603 aa
Sequence 3: BGUS 602 aa

```
HGUS    LGLQGGMLYPQESPSRECKELDGLWSFRADFSDNRRRGFEEQWYRRPLWESGPTVDMPVP 60
EGUS    ------MLRPVETPTREIKKLDGLWAFSLDREN---CGIDQRWWESALQESR---AIAVP 48
BGUS    ------MLYPINTETRGVFDLNGVWNFKLDYG----KGLEEKWYESKLTDT---ISMAVP 47
              ** * :: :*   .*:*:* *  *       *::::*:.  * ::     :.**
```

FIG. 13E

```
HGUS    SSFNDISQDWRLRHFVGWVWYEREVILPERWTQDLRTRVVLRIGSAHSYAIVWVNGVDTL 120
EGUS    GSFNDQFADADIRNYAGNVWYQREVFIPKGWAG---QRIVLRFDAVTHYGKVWVNNQEVM 105
BGUS    SSYNDIGVTKEIRNHIGYVWYEREFTVPAYLKD---QRIVLRFGSATHKAIVYVNGELVV 104
        .*:**      :*:. * ***:**. :*         *:***:::.   . *:**.  .:

HGUS    EHEGGYLPFEADISNLVQVGPLPSRLRITIAINNTLTPTTLPPGTIQYLTDTSKYPKGYF 180
EGUS    EHQGGYTPFEADVTPYVIAG---KSVRITVCVNNELNWQTIPPG--MVITDENGKKK--- 157
BGUS    EHKGGFLPFEAEINNSLRDG----MNRVTVAVDNILDDSTLPVG-LYSERHEEGLGKVIR 159
        **:**: ****::.  :  *     *:*:.::* *   *:* *      . .   *

HGUS    VQNTYFDFFNYAGLQRSVLLYTTPTTYIDDITVTTSVEQDS--GLVNYQISVKGSNLFKL 238
EGUS    -QSYFHDFFNYAGIHRSVMLYTTPNTWVDDITVVTHVAQDCNHASVDWQVVANG----DV 212
BGUS    -NKPNFDFFNYAGLHRPVKIYTTPFTYVEDISVVTDFNGPT--GTVTYTVDFQG-KAETV 215
         :.  .*******:.*.* :**** *:::**:*.*  .      . * : :  :*     :

HGUS    EVRLLDAENKVVANGTGTQGQLKVPGVSLWWPYLMHERPAYLYSLEVQLTAQTSLGPVSD 298
EGUS    SVELRDADQQVVATGQGTSGTLQVVNPHLWQP-----GEGYLYELCVTAKSQTEC----D 263
BGUS    KVSVVDEEGKVVASTEGLSGNVEIPNVILWEP-----LNTYLYQIKVELVNDGLT---ID 267
        .* : * : :***.  * .* ::: .  ** *       ***.: *    :       *

HGUS    FYTLPVGIRTVAVTKSQFLINGKPFYFHGVNKHEDADIRGKGFDWPLLVKDFNLLRWLGA 358
EGUS    IYPLRVGIRSVAVKGEQFLINHKPFYFTGFGRHEDADLRGKGFDNVLMVHDHALMDWIGA 323
BGUS    VYEEPFGVRTVEVNDGKFLINNKPFYFKGFGKHEDTPINGRGFNEASNVMDFNILKWIGA 327
        .*   .*:*:* *.  :**** ***** *..:***: :.*:**:    * *. :: *:**

HGUS    NAFRTSHYPYAEEVMQMCDRYGIVVIDECPGVGLAL----------P------QFFNNV 401
EGUS    NSYRTSHYPYAEEMLDWADEHGIVVIDETAAVGFNLSLGIGFEAGNKPKELYSEEAVNGE 383
BGUS    NSFRTAHYPYSEELMRLADREGLVVIDETPAVGVHLNFMATTGLGEGSERVSTWEKIR-- 385
        *:**:****:**:.  .*. *:***** ..**. *             .      : ..

HGUS    SLHHHMQVMEEVVRRDKNHPAVVMWSVANEPASHLESAGYYLKMVIAHTKSLDPS-RPVT 460
EGUS    TQQAHLQAIKELIARDKNHPSVVMWSIANEPDTRPQGAREYFAPLAEATRKLDPT-RPIT 442
BGUS    TFEHHQDVLRELVSRDKNHPSVVMWSIANEAATEEEGAYEYFKPLVELTKELDPQKRPVT 445
        : . * :.:.*:: ******:*****:***. :. :.*  *:  :   *:.***  **:*

HGUS    FVS--NSNYAADKGAPYVDVICLNSYYSWYHDYGHLELIQLQLATQFENWYKKYQ-KPII 517
EGUS    CVNVMFCDAHTDTISDLFDVLCLNRYYGWYVQSGDLETAEKVLEKELLAWQEKLH-QPII 501
BGUS    IVLFVMATPETDKVAELIDVIALNRYNGWYFDGGDLEAAKVHLRQEFHAWNKRCPGKPIM 505
         *     .  :*. :  .**:.** * .** : *.**  :  *  ::  * ::   :**:

HGUS    QSEYGAETIAGFHQDPPLMFTEEYQKSLLEQYHLGLDQKRRKYVVGELIWNFADFMTEQS 577
EGUS    ITEYGVDTLAGLHSMYTDMWSEEYQCAWLDMYHRVFD--RVSAVVGEQVWNFADFATSQG 559
BGUS    ITEYGADTVAGFHDIDPVMFTEEYQVEYYQANHVVFD--EFENFVGEQAWNFADFATSQG 563
         :***.:*:**:*.   . *:.****    :  *  :*  . . .***  ****** *.*.

HGUS    PTRVLGNKKGIFTRQRQPKSAAFLLRERYWKIAN-ET------- 613
EGUS    ILRVGGNKKGIFTRDRKPKSAAFLLQKRWTGMNFGEKPQQGGKQ 603
BGUS    VMRVQGNKKGVFTRDRKPKLAAHVFRERWTNIPDFGYKN----- 602
          ** *****:***:*:** **.::::*:  :
```

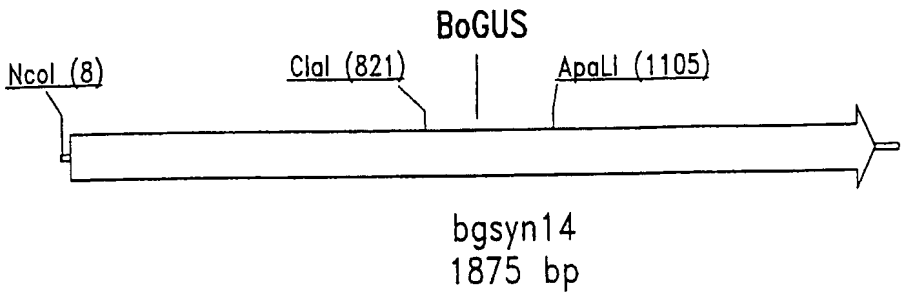


Fig. 14

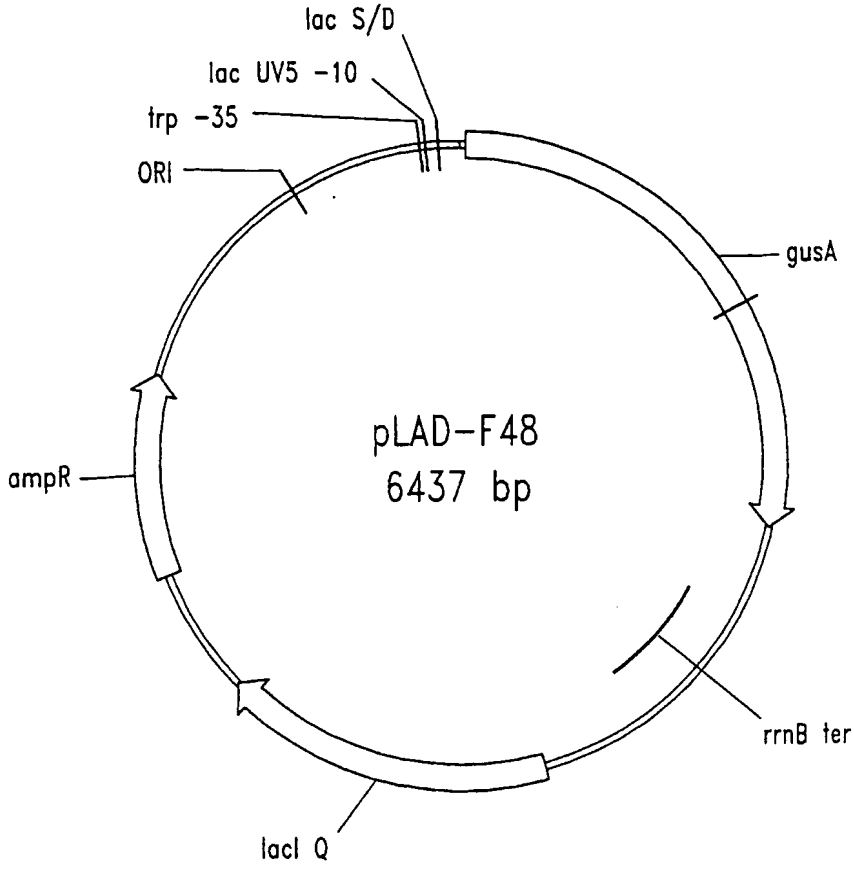

lac S/D
lac UV5 -10
trp -35
ORI
gusA
pLAD-F48
6437 bp
ampR
rrnB ter
lacI Q

Fig. 15